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13. ABSTRACT (Maximum 200 Words)

Our previous data have shown that I3C inhibits cell growth and induces apoptosis in cancer cells. We have also observed a reduction in the activated Akt and the activity of NF-κB in prostate cancer cells treated with I3C. We hypothesize that I3C/DIM functions as an inhibitor of NF-κB, which may be due to the inactivation of Akt-related signaling molecules, and ultimately leads to the induction of apoptotic processes. To test our hypotheses we investigated the effects of I3C/DIM on NF-κB and Akt activities in prostate cancer cells and non-tumorigenic prostate epithelial cells. We also investigated how the Akt and NF-κB pathways may cross-talk in prostate cancer cells exposed to I3C/DIM. We have found that I3C/DIM inhibited NF-κB activity and the inhibition was partly mediated through the inactivation of Akt. I3C and DIM inactivated NF-κB and Akt, regulated the expression of genes related to cell growth and apoptosis, and induced Bax translocation and cytochrome c release, resulting in inhibition of cell growth and induction of apoptosis in prostate cancer cells. However, no significant such effects were observed in non-tumorigenic CRL-2221 prostate epithelial cells, suggesting that I3C and DIM may be potent agents for the prevention and/or treatment of prostate cancer.

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Introduction

Epidemiological studies have shown an association between decreased prostate cancer risk and increased consumption of diet rich in indole-3-carbinol (I3C) which is readily converted to its dimeric product, 3,3'-diinolylmethane (DIM). The data from our laboratory and others have shown that I3C inhibits cell growth and induces apoptotic cell death in cancer cells by up-regulation of Bax and $p21^{WAF1}$, and down-regulation of Bcl-2/Bcl_{XL}. We have also found that I3C induces apoptosis in both androgen sensitive and androgen independent prostate cancer cells, suggesting that I3C/DIM may be a useful agent for prevention and/or treatment of hormone independent and metastatic prostate cancer for which there is no effective therapeutic strategy. We have also observed a drastic reduction in the activated form of the Akt, and DNA-binding activity of NF-κB in prostate cancer cells treated with I3C, suggesting a previously unknown mechanism of the action of I3C. These results provide important molecular evidence, which could be exploited for sensitization of prostate cancer cells to commonly available chemotherapeutic agents. Based on these results, we hypothesize that I3C/DIM functions as an inhibitor of NF-κB, which may be due to the inactivation of Akt-related signaling molecules that are important regulators of NF-κB DNA-binding activity, and ultimately leads to the induction of apoptotic processes. To test our hypotheses we will investigate how I3C/DIM inactivates NF-κB DNA binding activity and whether there are any differential effects of I3C/DIM between prostate cancer cells and non-tumorigenic prostate epithelial cells (CRL2221). We will also determine how I3C/DIM inhibits Akt activation leading to apoptotic cell death of prostate cancer cells, and investigate how the Akt and NF-κB pathways may cross-talk during the ultimate demise of prostate cancer cells induced by I3C/DIM. This will be investigated by critically evaluating the NF-κB upstream signaling molecules such as MEKK, MEK, NIK and IKK, which are known to play important roles in the activation of NF-κB. The results of this study will provide us with not only the information regarding the molecular mechanism(s) of action of I3C/DIM in prostate cancer cells, but will also provide us with molecular markers that may be useful for monitoring effectiveness of I3C/DIM during in vivo animal or human studies. In addition, these results should identify novel pathways, which could be targeted for the development of molecular therapeutic approaches for the prevention and/or treatment of prostate cancer in the future.

Body of Report

The original statement of work in the proposal is listed below:

Task-1: We will determine whether treatment of prostate epithelial cells with I3C/DIM elicit responses leading to modulation of NF-κB and investigate the molecular mechanism of NF-κB inactivation as proposed under specific aim-1. This investigation will be conducted using both I3C as well as DIM in androgen sensitive (LNCaP) and androgen independent (PC-3) prostate cancer cells and the data will be compared to those obtained from non-tumorigenic prostate epithelial cells (CRL2221). Task 1 will take 8 months to complete. Time Period 0-8 months.

Task-2: We will determine whether constitutive activation of Akt (by gene transfection studies) increases NF-κB activation in prostate epithelial cells as indicated under task-1, and thereby inhibits apoptotic processes induced by I3C/DIM. We will conduct transient transfection experiments followed by establishing stably transfected cells in the future in order to determine the inhibition of I3C/DIM induced cell death in those cells that over-express Akt and NF-κB. Task 2 will take 6-12 months to complete. Time Period 8-20 months.

Task-3: Once we complete task-1 and 2, we will start working on task 3, which will have significant number of transfection experiments. Task 3 will determine whether treatment of prostate epithelial cells with I3C/DIM will elicit responses that may determine causal association between the inactivation of Akt and NF-κB with the induction of apoptosis. Furthermore, we will investigate what signaling pathways are modified by I3C/DIM that leads to NF-κB inactivation. We will investigate different kinases that are involved in the NF-κB pathway, particularly the status of IKK, MAP kinase-MEK, MEKK1 and NIK. In order to establish cause and effect relationships of these genes with the ultimate effect of I3C/DIM, several transfection experiments are planned which will be very time consuming and labor intensive. Hence this task will take considerably more time. We expect to complete this task within 12 months. Time Period 20-32 months.

Task-4: Task-4 will be focused on to complete all data analysis, manuscript writing and for the development of novel ideas that may be submitted to Federal Agencies for further continued funding in a larger scale encompassing animal and human investigations to test (a) whether I3C/DIM could be an effective agent for the prevention and/or for the treatment of tumors in animal models, (b) whether I3C/DIM pre-exposure of prostate cancer cells will sensitize these cells to commonly available chemotherapeutic agents, and finally (c) whether I3C/DIM could be an effective agent for the prevention and/or treatment of human prostate cancer. Task 4 will be completed within the time period of 32-36 months.

We are now reporting the research accomplishments associated with each task outlined in the Statement of Work.

A. Our task-1 was focused on investigating the effects of I3C and DIM on modulation of NF-κB and the molecular mechanism(s) responsible for NF-κB inactivation, anti-proliferation,

and apoptosis caused by I3C and DIM in human prostate cancer cells. We investigated the effects of I3C on the regulation of cell growth, apoptosis induction, and NF-κB activity. We found that I3C inhibited cell growth, induced apoptosis, and inactivated NF-κB activity. These preliminary data have been included in the proposal submitted to DOD. After submission of the proposal, we continued to perform the experiments mentioned in Task 1 and therefore the following results are directly relevant to this progress report. Our continued investigation provided significant results. We have investigated the effect of DIM on NF-κB DNA binding activity. We found that DIM as an *in vivo* dimeric product of I3C also inhibited cell growth and NF-κB activity in prostate cancer cells. However, such effect was not observed in non-tumorigenic CRL-2221 prostate epithelial cells (Figure 1 and 2).

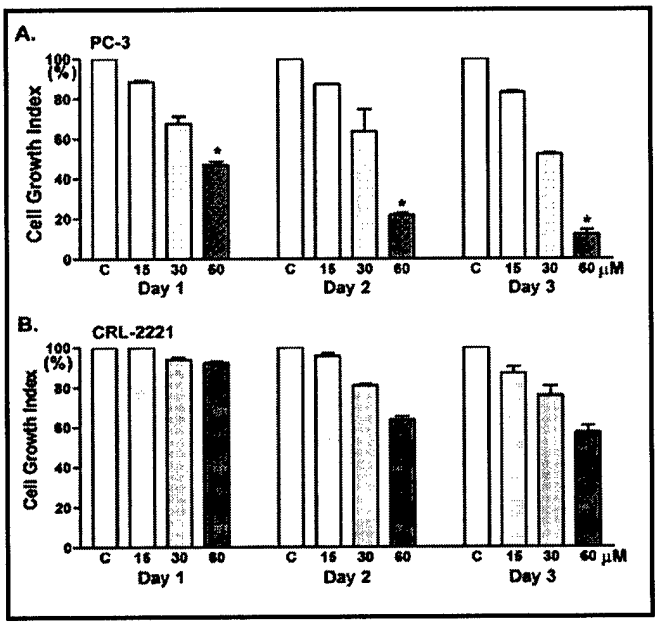

Figure 1: *MTT assay showing the significant inhibition of cell growth by DIM in PC3 cells (A) (*: $p<0.05$). Note that there is no significant growth inhibition in CRL-2221 cells (B).*

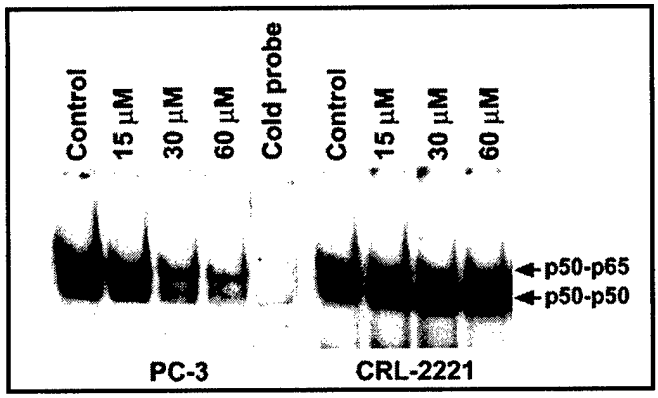

Figure 2: *Gel mobility shift assay showing that the NF-κB DNA binding activity is significantly inhibited by the treatment of DIM for 72 hours. Note that there is no change in NF-κB activity in DIM-treated CRL-2221 cells.*

To investigate the molecular mechanisms of I3C and DIM effects on NF-κB inactivation, anti-proliferation, and apoptosis, we utilized the high-throughput microarray, which can monitor the expression of twenty two thousands of genes simultaneously and rapidly, to determine the alternation of gene expression profiles of PC3 prostate cancer cells exposed to I3C and DIM. We have identified the genes that are involved in cell cycle arrest, apoptosis, transcription factor regulation, and other physiological processes induced by I3C and DIM treatment. We submitted a manuscript including these results to "The Journal of Nutrition". The manuscript was accepted for publication after we got the notice for funding from DOD, and was published in "The Journal of Nutrition" (1) after the date the funding began. Some parts of the results were also presented in

the 2003 AACR Annual Meeting (2). The summary of our results in the form of an abstract of our paper is presented below. Our published review article (3) is also attached to provide comprehensive results that we have obtained thus far, and therefore considered as our progress on this project.

Abstract: Studies from our laboratory and others have shown that indole-3-carbinol (I3C) and its *in vivo* dimeric product 3,3'-diindolylmethane (DIM) inhibit the growth of PC3 prostate cancer cells and induce apoptosis by inhibiting NF-κB and Akt pathways. In order to obtain a comprehensive gene expression profiles altered by I3C and DIM treated PC3 cells, we have utilized cDNA microarray to interrogate 22,215 known genes by using Affymetrix Human Genome U133A Array. We found a total of 738 genes which showed >2 fold change after 24 hours of DIM treatment. Among these genes, 677 genes were down-regulated and 61 genes were up-regulated. Similarly, 727 genes showed >2 fold changes in the expression with down-regulation of 685 genes and up-regulation of 42 genes in I3C treated cells. The altered expressions of genes were observed as early as 6 hours and were more evident with longer treatment. Upon cluster analysis, we found that both I3C and DIM up-regulated expression of genes that are related to the Phase I and Phase II enzymes, suggesting their increased capacity for detoxification of carcinogens or chemicals. We also found that I3C and DIM down regulated the expression of genes, which are critically involved in the regulation of cell growth, cell cycle, apoptosis, signal transduction, Pol II transcription factor, and oncogenesis. Real-time RT-PCR analysis was conducted to confirm the data of cDNA microarray, and the results were consistent with the microarray data. From these results, we conclude that I3C and DIM caused changes in the expression of a large number of genes that are related to the control of carcinogenesis, cell survival, and physiological behaviors. This may provide molecular mechanism(s) by which I3C and DIM exert their pleiotropic effects on PC3 prostate cancer cells and these information could be further exploited for devising chemopreventive and/or therapeutic strategies for prostate cancer.

Conclusion: The task-1 has been fully completed, which was initiated during the submission and successful funding of this proposal.

B. Our task-2 was to investigate the relationship between Akt and NF-κB, and the effects of I3C and DIM on Akt and NF-κB pathways. By immunoprecipitation, kinase assay, and Western Blot analysis, we have found that I3C inhibits Akt activity in PC3 prostate cancer cells. These preliminary data was included in our proposal at the time of submission. By Western Blot analysis, we also found that DIM inhibited phosphorylation of Akt (Figure 3).

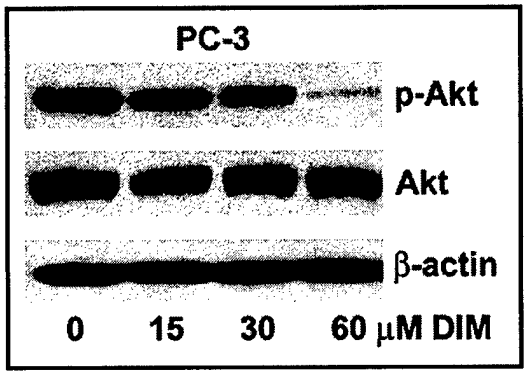

Figure 3: *Western Blot analysis showing that DIM treatment for 72 hours significantly inhibits the phosphorylation of Akt in PC3 prostate cancer cells.*

To investigate the relationship between Akt and NF-κB, we have conducted transfection

experiments with Akt (wild, mutant, or constitutively activated) expression vector and NF-κB-Luc vector that contains NF-κB binding and reporter sequences, and measured the activity of luciferase as well as NF-κB DNA binding activity in transfected prostate cancer cells. We have also detected the effects of DIM on Akt and NF-κB in transfected and un-transfected prostate cancer cells. We found that Akt transfection up-regulated activity of NF-κB as demonstrated by luciferase assay (Figure 4) and EMSA (Figure 5). We also found that DIM inhibited the activation of NF-κB induced by Akt transfection (Figure 4 and 5), suggesting that the inhibition of NF-κB by DIM is partly mediated by Akt pathway.

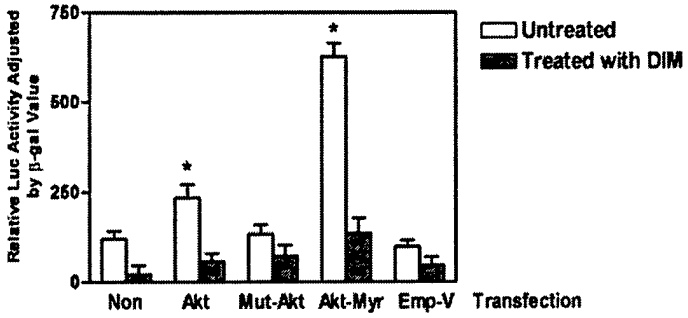

Figure 4: *Luciferase activity in transfected PC3 cells with or without DIM treatment. (Non: no transfection; Akt: transfected with wild Akt; Mut-Akt: transfected with mutant Akt; Akt-Myr: transfected with constitutively activated Akt; Emp-v: transfected with empty vector; *: $p<0.05$; $n=2$)*

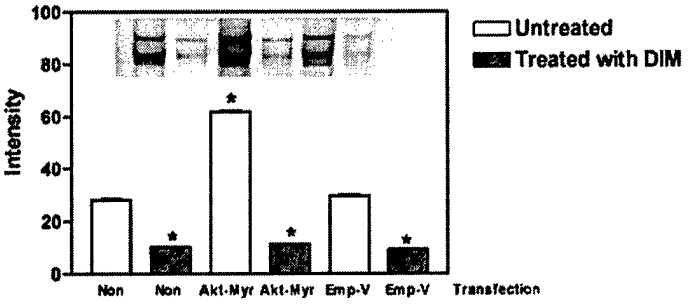

Figure 5: *EMSA and densitometric analysis of NF-κB DNA-binding activity in transfected PC3 cells with or without DIM treatment. (Non: no transfection; Akt-Myr: transfected with constitutively activated Akt; Emp-V: transfected with empty vector; *: $p<0.05$; $n=2$)*

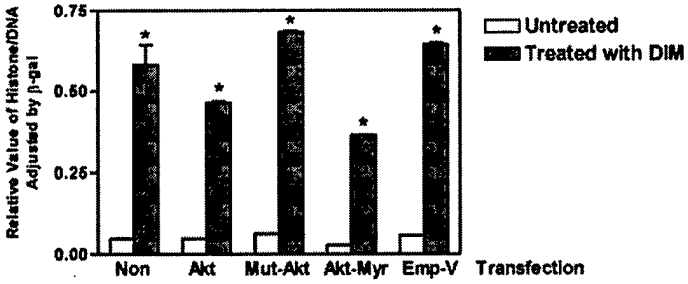

Figure 6: *Induction of apoptosis in DIM treated PC3 cells tested by ELISA. (Non: no transfection; Akt: transfected with wild Akt; Mut-Akt: transfected with mutant Akt; Akt-Myr: transfected with constitutively activated Akt; Emp-v: transfected with empty vector; *: $p<0.05$; $n=2$)*

We have also investigated the pro-apoptotic effect of DIM in PC3 prostate cancer cells and Akt transfected PC3 cells. We found that DIM induced apoptosis in both parental and Akt transfected PC3 cancer cells (Figure 6). However, Akt transfection showed inhibitory effect on the apoptosis induced by DIM, suggesting that the pro-apoptotic effect of DIM is mediated by Akt and NF-κB pathways. We also investigated the effect of DIM on the expression of genes related to the control of cell growth and apoptosis. The results of our study have been submitted

to the journal "Frontiers in Bioscience" and the manuscript has been accepted for publication (4). The summary of our results in the form of an abstract is presented below.

Abstract: Prostate cancer is the second leading cause of cancer related deaths in men in the United States. I3C and its *in vivo* dimeric product, DIM, have been found to inhibit the growth of prostate cancer cells. However, the molecular mechanism(s) by which DIM elicits its effects on prostate cancer cells has not been fully elucidated. We have previously shown that I3C induces apoptosis and inhibits the activation of NF-κB pathway, which could be mediated via Akt signaling pathway. In this study, we investigated whether there is any cross-talk between Akt and NF-κB during DIM-induced apoptosis in PC-3 prostate cancer cells. We found that DIM inhibited cell growth and induced apoptosis in PC-3 prostate cancer cells but not in non-tumorigenic CRL2221 human prostate epithelial cells. DIM also inhibited EGFR expression, PI3K kinase activity, and Akt activation, and abrogated the EGF-induced activation of PI3K in prostate cancer cells. NF-κB DNA-binding analysis and transfection studies with Akt cDNA constructs revealed that Akt transfection resulted in the induction of NF-κB activity and this was inhibited by DIM treatment. DIM treatment also showed significant induction of apoptosis in non-transfected cells compared to Akt and Akt-Myr transfected prostate cancer cells. From these results, we conclude that the inhibition of Akt and NF-κB activity and their cross-talk is a novel mechanism by which DIM inhibits cell growth and induces apoptotic processes in prostate cancer cells but not in non-tumorigenic prostate epithelial cells.

Conclusion: We have completed majority parts of task-2 and plan to complete this task in the second year.

C. Our task-3 is to investigate the molecular mechanisms of I3C and DIM effects on NF-κB and Akt pathways by transfection and kinase assays, parts of which have been completed as stated above. We have now started to conduct the transfection experiments with IKK, NIK, MEK, MEKK plasmids to establish cause and effect relationships of those molecules with the effect of I3C/DIM in prostate cancer cells.

Conclusion: We plan to complete task-3 in the second and third year.

D. Once the tasks 1-3 are all completed, then we will make progress on task-4 to complete all experiments, data analysis and manuscript writing during the third year of funding.

Key Research Accomplishments

- Detected the effect of I3C and DIM on NF-κB DNA binding activity in prostate cancer cells and non-tumorigenic CRL-2221 prostate epithelial cells by EMSA.
- Detected the apoptosis induced by I3C and DIM in prostate cancer cells. Measuring the protein level of genes (Akt, pAkt, Bcl_{XL}, $p21^{WAF1}$, EGFR, etc) related to the control of cell growth and apoptosis in prostate cancer cells.
- Gene expression profiling of I3C and DIM treated prostate cancer cells by microarray analysis. Cluster analysis of the expression of genes related to NF-κB inactivation, cell cycle arrest, apoptosis, transcript regulation, and other physiological processes in prostate cancer cells exposed to I3C and DIM.
- Determined the effects of I3C and DIM on Akt kinase activity in PC3 prostate cancer cells and non-tumorigenic CRL-2221 prostate epithelial cells by immunoprecipitation, kinase assay, and Western Blot analysis.
- Detected the cross-talk between Akt and NF-κB in PC3 prostate cancer cells by transfection, luciferase assay, and EMSA.
- Determined the effects of DIM on Akt and NF-κB pathways in Akt transfected PC3 prostate cancer cells by transfection, luciferase assay, and EMSA.
- Determined the effects of DIM on the induction of apoptosis in Akt transfected PC3 prostate cancer cells by transfection, ELISA, and Western Blot analysis.

Reportable Outcomes

1. Li Y, Li X, Sarkar FH. Gene expression profiles of I3C and DIM treated PC3 human prostate cancer cells by cDNA microarray analysis. J Nutr. 133(4):1011-1019, 2003.
2. Li Y, Li X, Sarkar FH. Gene expression profiles of I3C and DIM treated PC3 human prostate cancer cells by cDNA microarray analysis. Proc Am Assoc Cancer Res 44:1501 abstr 6551, 2003.
3. Sarkar FH, Li Y. Significance of indole-3-carbinol and its metabolite in human cancers. Evidence-Based Integrative Medicine 1:33-41, 2003.
4. Li Y, Chinni SR, Sarkar FH. Selective growth regulatory and pro-apoptotic effects of DIM is mediated by Akt and NF-κB pathways in prostate cancer cells. Frontiers in Bioscience (In press)

Conclusions

We have found that I3C and DIM inhibit NF-κB DNA binding activity in prostate cancer cells. The inhibition of NF-κB by I3C and DIM is partly mediated through the inactivation of Akt. I3C and DIM inhibit phosphorylation of Akt, inactivate NF-κB, and regulate the expression of genes related to the control of cell growth and apoptosis, resulting in inhibition of cell growth and induction of apoptosis in prostate cancer cells. However, no significant such effects were observed in non-tumorigenic CRL-2221 prostate epithelial cells, suggesting that I3C and DIM may be potent agents for the prevention and/or treatment of prostate cancer. The task-1 has been fully completed. We have completed certain parts of task-2. We plan to complete task-2 and begin working on task-3 in the second year. We plan to complete task-3 and task-4 in the third year as originally planned.

References

1. Li Y, Li X, Sarkar FH. Gene expression profiles of I3C and DIM treated PC3 human prostate cancer cells by cDNA microarray analysis. J Nutr. 133(4):1011-1019, 2003.
2. Li Y, Li X, Sarkar FH. Gene expression profiles of I3C and DIM treated PC3 human prostate cancer cells by cDNA microarray analysis. Proc Am Assoc Cancer Res 44:1501 abstr 6551, 2003.
3. Sarkar FH, Li Y. Significance of indole-3-carbinol and its metabolite in human cancers. Evidence-Based Integrative Medicine 1:33-41, 2003.
4. Li Y, Chinni SR, Sarkar FH. Selective growth regulatory and pro-apoptotic effects of DIM is mediated by Akt and NF-κB pathways in prostate cancer cells. Frontiers in Bioscience (In press)

Appendices

Publications and abstracts during the first year of funding:

1. Li Y, Li X, Sarkar FH. Gene expression profiles of I3C and DIM treated PC3 human prostate cancer cells by cDNA microarray analysis. J Nutr. 133(4):1011-1019, 2003.
2. Li Y, Li X, Sarkar FH. Gene expression profiles of I3C and DIM treated PC3 human prostate cancer cells by cDNA microarray analysis. Proc Am Assoc Cancer Res 44:1501 abstr 6551, 2003.
3. Sarkar FH, Li Y. Significance of indole-3-carbinol and its metabolite in human cancers. Evidence-Based Integrative Medicine 1:33-41, 2003.
4. Li Y, Chinni SR, Sarkar FH. Selective growth regulatory and pro-apoptotic effects of DIM is mediated by Akt and NF-κB pathways in prostate cancer cells. Frontiers in Bioscience (In press)

Gene Expression Profiles of I3C- and DIM-Treated PC3 Human Prostate Cancer Cells Determined by cDNA Microarray Analysis[1]

Yiwei Li, Xingli Li and Fazlul H. Sarkar[2]

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ABSTRACT Studies from our laboratory and others have shown that indole-3-carbinol (I3C) and its in vivo dimeric product, 3,3′-diindolylmethane (DIM), inhibit the growth of PC3 prostate cancer cells and induce apoptosis by inhibiting nuclear factor (NF)-κB and Akt pathways. To obtain comprehensive gene expression profiles altered by I3C- and DIM-treated PC3 cells, we utilized cDNA microarray to interrogate the expression of 22,215 known genes using the Affymetrix Human Genome U133A Array. We found a total of 738 genes that showed a greater than twofold change after 24 h of DIM treatment. Among these genes, 677 genes were down-regulated and 61 were up-regulated. Similarly, 727 genes showed a greater than twofold change in expression, with down-regulation of 685 genes and up-regulation of 42 genes in I3C-treated cells. The altered expressions of genes were observed as early as 6 h and were more evident with longer treatment. Upon cluster analysis, we found that both I3C and DIM up-regulated the expression of genes that are related to the Phase I and Phase II enzymes, suggesting their increased capacity for detoxification of carcinogens or chemicals. We also found that I3C and DIM down-regulated the expression of genes that are critically involved in the regulation of cell growth, cell cycle, apoptosis, signal transduction, Pol II transcription factor and oncogenesis. Real-time reverse transcription-polymerase chain reaction analysis was conducted to confirm the cDNA microarray data, and the results were consistent. We conclude that I3C and DIM affected the expression of a large number of genes that are related to the control of carcinogenesis, cell survival and physiologic behaviors. This may help determine the molecular mechanism(s) by which I3C and DIM exert their pleiotropic effects on PC3 prostate cancer cells; in addition, this information could be further exploited for devising chemopreventive and/or therapeutic strategies for prostate cancer. J. Nutr. 133: 1011–1019, 2003.

KEY WORDS: • *indole-3-carbinol* • *3,3′-diindolylmethane* • *gene expression* • *microarray* • *prostate cancer cells.*

Epidemiologic studies have shown that a high dietary intake of fruits and vegetables protects against carcinogenesis in many tissues (1,2). Among vegetables with anticarcinogenic properties, the cruciferous vegetable family including broccoli, cabbage, brussels sprouts and cauliflower appears to be most effective at reducing the risk of cancers (3,4). Indole-3-carbinol (I3C),[3] a common phytochemical in the human diet, is present in almost all members of the cruciferous vegetable family. There is growing evidence showing that I3C has the potential to prevent or even treat a number of common cancers, especially those that are hormone-related (5,6). It has been reported that a diet rich in cruciferous vegetables or supplements of I3C caused regression of tumors or decreased the rate of growth in patients with recurrent laryngeal papillomatosis (7,8). The in vivo and in vitro studies have also demonstrated that I3C possesses anticarcinogenic effects in experimental animals and inhibits the growth of human cancer cells (9,10). Because of this information, interest in I3C as a cancer chemopreventive agent has increased greatly in the last few years.

I3C is chemically unstable in acidic environments and is rapidly converted in the stomach to a variety of condensation products. Among them, 3,3′-diindolylmethane (DIM) is a major acid condensation product of I3C in vitro and in vivo. Because of the ready conversion of I3C to DIM and other products under a variety of biological conditions, the biological effects of I3C may be attributable to both I3C and DIM. Experimental studies have revealed that DIM exhibits inhibitory effects on cancer cells by inhibiting cell growth and inducing apoptosis that are similar to the effects observed with I3C (11,12). It has been reported that DIM exerts its chemoprotective effects in estrogen-responsive tissues, and DIM-induced G_1 arrest occurs with up-regulation of $p21^{WAF1/CIP1}$ in

[1] Funded in part by a grant to F.H.S. from the Department of Defense.
[2] To whom correspondence should be addressed.
E-mail: fsarkar@med.wayne.edu.
[3] Abbreviations used: ATF, activating transcription factor; CBFβ, core binding factor β; CYP, cytochrome P_{450}; DIM, 3,3′-diindolylmethane; DMSO, dimethyl sulfoxide; EGFR, epidermal growth factor receptor; FGF, fibroblast growth factor; I3C, indole-3-carbinol; MAPK, mitogen-activated protein kinase; MAP2K, MAP kinase kinase; MIG, mitogen-inducible gene; NF, nuclear factor; PI3K, phosphatidylinositol-3-kinase; RT-PCR, reverse transcription-polymerase chain reaction; ST16, suppressor of tumorigenicity 16; TFDP, transcription factor Dp-1; TGF-β, transforming growth factor-β.

Manuscript received 20 November 2002. Initial review completed 29 December 2002. Revision accepted 21 January 2003.

breast cancer cells, suggesting its inhibitory effects on hormone-related cancers (11,13).

Prostate cancer is the most common nondermatological carcinoma in the United States with an estimated 189,000 new cases and 30,200 deaths in 2002 (14). Up to 30% of men undergoing radical prostatectomy will relapse, often as a result of micrometastatic disease present at the time of surgery (15). Thus, there is a tremendous need for the development of mechanism-based and targeted strategies for prevention and treatment of prostate cancer. cDNA microarray analysis allows us to examine the expression of tens of thousands of genes that can be monitored simultaneously and rapidly and, in turn, provides an opportunity to determine the effects of anticancer agents on prostate cancer cells (16). The gene expression profiles of various types of cancers were analyzed using cDNA microarray (17,18). The alterations of gene expression profiles by several anticancer agents have also been reported (19,20). This information is likely to contribute to devising preventive and/or therapeutic strategies more accurately, and will help to determine the molecular mechanism(s) of action of chemopreventive and/or therapeutic agents.

Our previous studies showed that I3C inhibits the growth of PC3 prostate cancer cells and induces apoptosis by inhibiting the nuclear factor (NF)-κB and Akt signaling pathways, suggesting that I3C may serve as a preventive and/or therapeutic agent against prostate cancer (21,22). However, little is known about the global gene expression profiles of prostate cancer cells after I3C and DIM treatment; the precise molecular mechanism(s) by which I3C and DIM exert their tumor suppressive effects on prostate cancer is also unclear. In this study, we utilized the high throughput gene chip, which contains 22,215 known genes, to identify changes in gene expression in PC3 prostate cancer cells exposed to I3C and DIM.

MATERIALS AND METHODS

Cell culture and growth inhibition. PC3 human prostate cancer cells (ATCC, Manassas, VA) were cultured in RPMI-1640 media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin in a 5% CO_2 atmosphere at 37°C. I3C (Sigma, St. Louis, MO) or DIM (LKT, St. Paul, Minnesota) was dissolved in dimethyl sulfoxide (DMSO) to make a 100 or 50 mmol/L stock solution, respectively. For growth inhibition, PC3 cells were treated with 30, 60 and 100 μmol/L I3C or 15, 30, and 60 μmol/L DIM for 1–3 d. Control PC3 cells received 0.1% DMSO for the same time periods. The cells were then incubated with MTT (0.5 g/L, Sigma, St. Louis, MO) at 37°C for 4 h and with DMSO at room temperature for 1 h. The spectrophotometric absorbance of the samples was measured using the ULTRA Multifunctional Microplate Reader (TECAN, Durham, NC) at 495 nm. The experiment was repeated three times and *t* tests were performed to determine whether cell growth was inhibited by the treatments.

cDNA microarray analysis. PC3 cells were treated with 60 μmol/L I3C or 40 μmol/L DIM for 6, 24 and 48 h. DIM is the in vivo dimeric product of I3C. The doses of I3C and DIM chosen for microarray experiment were close to the 50% inhibitory concentration. However, the biological relevance of these doses in relation to prevention or therapy has not been fully evaluated, although they may have therapeutic application. The rationale for choosing these time points was to capture the expression profiles of early-response genes, genes that may be involved in the onset of growth inhibition and apoptotic processes and, finally, genes that may act as performers for the induction of apoptosis. Total RNA from each sample was isolated by Trizol (Invitrogen, Carlsbad, CA) and purified using the RNeasy Mini Kit and RNase-free DNase Set (QIAGEN, Valencia, CA) according to the manufacture's protocols. cDNA for each sample was synthesized using a Superscript cDNA Synthesis Kit (Invitrogen) and a T7-$(dT)_{24}$ primer instead of the oligo(dT) provided in the kit. Then, the biotin-labeled cRNA was transcribed in vitro from cDNA using a BioArray HighYield RNA Transcript Labeling Kit (ENZO Biochem, New York, NY) and purified using the RNeasy Mini Kit. The purified cRNA was fragmented by incubation in fragmentation buffer (200 mmol/L Tris-acetate pH 8.1, 500 mmol/L potassium acetate, 150 mmol/L magnesium acetate) at 95°C for 35 min and chilled on ice. The fragmented labeled cRNA was applied to the Human Genome U133A Array (Affymetrix, Santa Clara, CA), which contains 22,215 human gene cDNA probes, and hybridized to the probes in the array. After washing and staining, the arrays were scanned using a HP GeneArray Scanner (Hewlett-Packard, Palo Alto, CA). Two independent experiments were performed to verify the reproducibility of results.

Microarray data normalization and analysis. The gene expression levels of samples were normalized and analyzed using Microarray Suite, MicroDB and Data Mining Tool software (Affymetrix). The signal value of the experimental array was multiplied by a normalization factor to make its mean intensity equivalent to the mean intensity of the control array using Microarray Suite software according to manufacturer's protocol. The absolute call (present, marginal, absent) and average difference of 22,215 gene expressions in a sample, and the absolute call difference, fold change and average difference of gene expressions between two or several samples were identified using the above-mentioned software. Statistical analysis of the difference in the mean expression of genes that indicated a greater than twofold change was performed repeatedly between treated and untreated samples using *t* tests. Average-linkage hierarchical clustering of the data was applied using the Cluster (23) and the results were displayed with TreeView (23). The genes showing altered expression were also categorized on the basis of their location, cellular component and reported or suggested biochemical, biological and molecular functions using Onto-Express (24). Genes that were not annotated or not easily classified were excluded from the functional clustering analysis.

Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis. The total RNA prepared for microarray analysis was also used for RT-PCR analysis of selected genes. Total RNA (2 μg) from each sample was subjected to reverse transcription using a Superscript first strand cDNA synthesis kit (Invitrogen) according to the manufacturer's protocol. Real-time PCR reactions were then

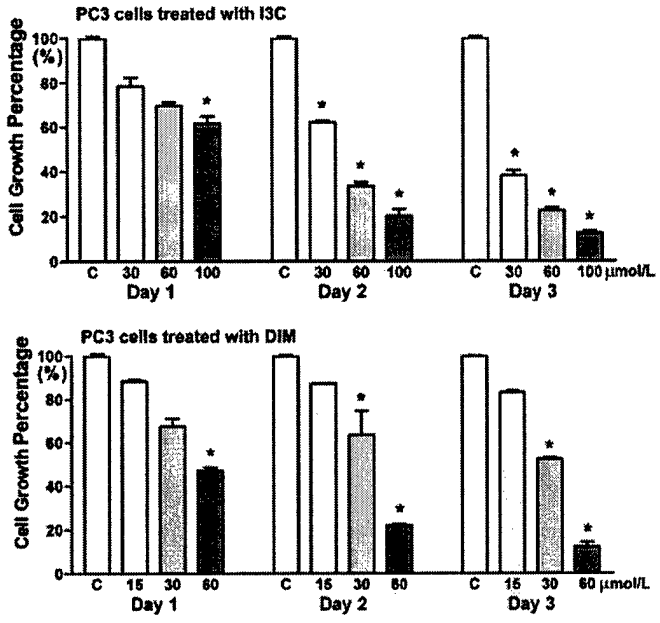

FIGURE 1 Effects of various doses of indole-3-carbinol (I3C) and 3,3'-diindolylmethane (DIM) on the growth of PC3 cells for 3 d. Data are means ± SEM, $n = 3$. *Different from solvent control (C), $P < 0.05$.

carried out in a 25 μL reaction mixture (2 μL of cDNA, 12.5 μL of 2X SYBR Green PCR Master Mix, 1.5 μL of 5 μmol/L specific gene primer pair and 9 μL of H_2O) in an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). The sequences of primers used in the real-time PCR reaction were described previously (25). The PCR program was initiated by 2 min at 50°C and 10 min at 95°C before 40 thermal cycles, each of 15 s at 95°C and 1 min at 60°C. Data were analyzed according to the comparative cycle threshold (Ct) method and were normalized by actin expression in each sample. Melting curves for each PCR reaction were generated to ensure the purity of the amplification product.

RESULTS

Cell growth inhibition. The MTT assay showed that the treatment of PC3 prostate cancer cells with I3C or DIM resulted in dose- and time-dependent inhibitions of cell proliferation (**Fig. 1**), demonstrating the growth inhibitory effect of these compounds on PC3 cells. These results are consistent with our previously published results (22).

Regulation of mRNA expression. The gene expression profiles of PC3 cells treated with I3C or DIM were assessed

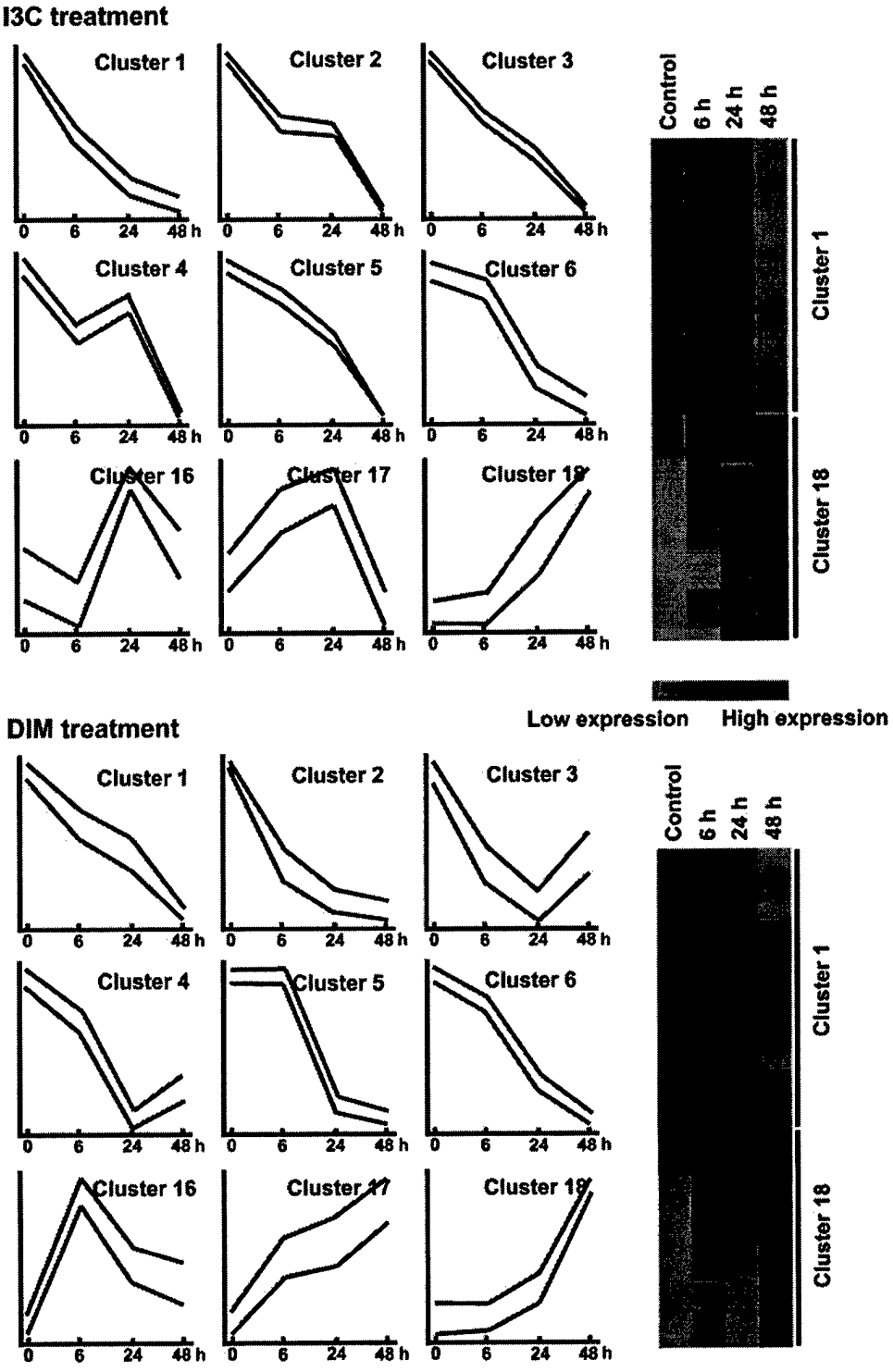

FIGURE 2 Cluster analysis of genes showing alterations in mRNA expression after indole-3-carbinol (I3C) and 3,3′-diindolylmethane (DIM) treatment of PC3 cells. Cluster 1 and cluster 18 included the genes showing typical gradual decreases and increases in expression, respectively.

using cDNA microarray. Two independent experiments showed a total of 738 genes showing a greater than twofold change after 24 h of DIM treatment. Among these, 677 genes were down-regulated and 61 were up-regulated. Similarly, 727 genes showed a greater than twofold change in expression with down-regulation of 685 genes and up-regulation of 42 genes in I3C-treated cells. Clustering analysis showed 18 different types of expression alterations in DIM- or I3C-treated PC3 cells, respectively. Cluster 1 and cluster 18 included the genes showing typical gradual decreases and increases in expression, respectively. The altered expressions of genes occurred after only 6 h of I3C and DIM treatment, and were more evident with longer treatment (**Fig. 2**).

The altered genes were also subjected to cluster analysis according to location and cellular components. The genes showing altered expression were located primarily on chromosomes 1, 2, 3, 6, 7 and 12, and were responsible mainly for the transcription and translation of components of the nucleus and integral plasma membrane proteins. After clustering based on biological function, we found that I3C and DIM down-regulated mainly genes that are involved in the processes of signal transduction, oncogenesis, cell proliferation, antiapoptosis and transcription regulation from Pol II promoter. On the other hand, I3C and DIM up-regulated mainly genes that are related to Phase I and II biotransformation, signal transduction, induction of apoptosis, inflammation response and prostaglandin metabolism (**Tables 1, 2 and 3**). When genes were classified by molecular function, I3C and DIM treatment

TABLE 1

Classification by function of genes with altered expression in PC3 cells treated with 60 μmol/L indole-3-carbinol (I3C) for 48 h[1]

Down-regulation	Genes, *n*	Up-regulation	Genes, *n*
Biological processes			
Signal transduction	56	Cell cycle checkpoint	3
Transcription regulation from Pol II promoter	18	Developmental processes	3
Cell adhesion	16	Protein phosphorylation	2
Cell proliferation	16	Small molecule transport	2
Oncogenesis	12	Central nervous system development	2
Cell cycle control	11	Signal transduction	2
Protein phosphorylation	10	Nonselective vesicle transport	1
Cell-cell signaling	8	Skeletal development	1
Protein modification	8	Immune response	1
RNA processing	8	Cell surface receptor linked signal transduction	1
Transcription regulation	6	Cell growth and maintenance	1
DNA replication	5	Stress response	1
Positive control of cell proliferation	5	Sodium transport	1
Receptor tyrosine phosphatase signaling	5	Cytostolic calcium ion concentration elevation	1
Transcription from Pol II promoter	5	G-protein linked receptor protein signaling	1
Drug resistance	4	Repression of transcription from Pol II promoter	1
EGF receptor signaling pathway	4	Induction of apoptosis	1
Translational regulation, initiation	4	Excretion	1
JNK cascade	4	Apoptosis	1
Cell motility	3	Spermatid development	1
JAK-STAT cascade	2	Male meiosis	1
Antiapoptosis	2	Peripheral nervous system development	1
Activation of JUN kinase	2	Antimicrobial humoral response	1
TGFβ receptor signaling pathway	2	Cysteine metabolism	1
MAP3K cascade	1	Brain development	1
Molecular functions			
Transcription factor	13	Calcium binding	2
DNA binding	12	Serpin	2
Protein serine/Threonine kinase	12	Cytochrome P_{450}	1
Adenosinetriphosphatase	9	Tumor suppressor	1
Transcription co-activator	9	Serine C-palmitoyltransferase	1
Protein kinase	9	Receptor signaling protein	1
RNA binding	8	Interleukin receptor	1
RNA polymerase II transcription factor	6	Diamine *N*-acetyltransferase	1
Transcription co-repressor	5	Receptor	1
Translation factor	5	Ligand	1
Signal transduction	3	G-protein linked receptor	1
Receptor protein tyrosine phosphatase	3	Protein binding	1
Transcription activating factor	3	Transcription co-repressor	1
Apoptosis inhibitor	2	Tryptophan transporter	1
JAK pathway signal transduction adaptor	2	Purine nucleotide transporter	1
Epidermal growth factor receptor	2	ATP-binding cassette (ABC) transporter	1
MAP3K	2	ATP binding	1
Inositol-1,4,5-triphosphate receptor	2	Transporter	1
Type II TGFβ receptor	2	Enzyme inhibitor	1
Cytokine	2	Heat shock protein	1
Cell cycle regulator	1	Chaperone	1
Transcription co-factor	1	Ligand-dependent nuclear receptor	1

[1] Abbreviations used: EGF, epidermal growth factor; JNK, c-Jun N-terminal protein kinase; MAPK, mitogen-activated protein kinase; TGF, transforming growth factor.

TABLE 2

Classification by function of genes with altered expression in PC3 cells treated with 40 μmol/L 3,3'-diindolylmethane (DIM) for 48 h[1]

Down-regulation	Genes, *n*	Up-regulation	Genes, *n*
Biological processes			
Signal transduction	42	Inflammatory response	4
Oncogenesis	16	Signal transduction	4
Cell proliferation	16	Prostaglandin metabolism	2
Cell-cell signaling	15	Cell cycle checkpoint	2
Cell cycle control	13	G-protein linked receptor protein signaling	2
Transcription regulation from Pol II promoter	11	Protein complex assembly	1
Transcription from Pol II promoter	11	Immune response	1
RNA processing	9	Potassium transport	1
Protein phosphorylation	9	Defense response	1
Cell adhesion	9	Physiological processes	1
Cell motility	9	Response to pathogenic fungi	1
Positive control of cell proliferation	6	Proteolysis and peptidolysis	1
DNA replication	5	Complement activation	1
RNA splicing	5	Glucose metabolism	1
Protein complex assembly	5	Developmental processes	1
Pathogenesis	5	Stress response	1
Cytostolic calcium ion concentration	5	Homophilic cell adhesion	1
Antiapoptosis	5	Small molecule transport	1
Immune response	5	Heavy metal response	1
Transcription from Pol I promoter	3	Proton transport	1
JNK cascade	2	Virulence	1
Transcription	2	Receptor protein tyrosine kinase signaling	1
Transcription from Pol III promoter	2	Phosphatidylinositol bisphosphate hydrolysis	1
EGF receptor signaling pathway	1	Cytostolic calcium ion concentration elevation	1
Transcription initiation from Pol II promoter	1	Circulation	1
Activation of MAPK	1	Cell surface receptor linked signal transduction	1
Molecular functions			
RNA binding	20	Cytochrome P_{450}	3
Transcription factor	16	Electron transporter	3
DNA binding	15	Protein binding	3
Transcription co-activator	10	Calcium binding	2
RNA polymerase II transcription factor	9	Cystine/glutamate antiporter	1
Protein binding	8	Chaperone	1
Signal transduction	8	Interleukin-1 receptor	1
Protein serine/Threonine kinase	8	Calcium-activated potassium channel	1
Adenosinetriphosphatase	7	Prostaglandin-endoperoxide synthase	1
Cytokine	7	GTP binding	1
Transcription activating factor	6	Serine-type peptidase	1
Heat shock protein	6	Complement factor D	1
Cell cycle regulator	3	Double-stranded RNA binding	1
Specific RNA pol II transcription factor	3	Adenosine deaminase	1
Apoptosis inhibitor	3	RNA binding	1
Transforming growth factorβ receptor	3	Aldo-keto reductase	1
General RNA pol II transcription factor	2	Phosphoenolpyruvate carboxykinase	1
G_1/S-specific cyclin	2	Tumor suppressor	1
Tumor antigen	2	Adenosinetriphosphatase	1
Receptor protein tyrosine phosphatase	2	General RNA polymerase II transcription factor	1
Cyclin	2	Bradykinin receptor	1
Growth factor	1	Chemokine receptor	1
Interleukin-8 receptor ligand	1	Cytokine	1
MAP kinase kinase	1	Enzyme	1

[1] See Table 1 for abbreviations.

down-regulated those responsible for RNA binding, DNA binding, transcription factors, transcription coactivators and protein kinases including mitogen-activated protein kinase (MAPK), MAP2kinase, MAP3kinase, c-Jun N-terminal protein kinase and up-regulated tumor suppressors, transcription corepressors and cytochrome P_{450} enzymes (Tables 1–3). However, I3C and DIM also showed some differential effects on gene expression profiles (Table 3).

Target verification by RT-PCR. To verify the alterations of gene expression at the mRNA level, which appeared on the microarray, we chose six genes [transforming growth factor-β (TGF-β)2, $p57^{KIP2}$, cytochrome b5, eukaryotic protein synthesis initiation factor 4, thrombospondin 1 and neuropilin 1] with varying expression profiles for real-time RT-PCR analysis. The results of real-time RT-PCR analysis for these selected genes were consistent with the microarray data (**Figs. 3** and **4**). Gene expression alterations were similar by real-time RT-PCR analysis, although the fold changes in the expression level differed somewhat in the two analytical methods. These results support the findings obtained from microarray experiments, and also suggest that I3C and DIM regulate the transcription of genes that are

TABLE 3

Fold changes of specific genes in PC3 cells treated with 60 μmol/L indole-3-carbinol (I3C) or 40 μmol/L 3,3'-diindolylmethane (DIM) for 6, 24 or 48 h[1,2]

Gene name	I3C			DIM		
	6 h	24 h	48 h	6 h	24 h	48 h
Phase I and II enzymes						
NM_000499.2 Cytochrome P_{450} (CYP1A1)	1.4	3.7	5.2	1.8	2.2	5.4
M25813.1 Human P_{450}c21 gene	NC[3]	NC	NC	NC	NC	3.5
N21019 Cytochrome P_{450}	NC	NC	NC	NC	5.2	3.5
AU154504 Cytochrome P_{450}	NC	NC	NC	NC	2.3	3.4
AU144855 Cytochrome P_{450}	NC	NC	NC	NC	2.3	3
NM_000104.2 Cytochrome P_{450} (CYP1B1)	NC	NC	NC	2	1.9	2.2
AB018580.1 Aldo-keto reductase	NC	NC	3.6	NC	2.9	9.2
NM_005589 Methylmalonate-semialdehyde dehydrogenase	NC	NC	NC	NC	NC	2.4
AK000550.1 Phospholipase A_2 β	NC	NC	NC	NC	5	NC
NM_000853.1 Glutathione *S*-transferase theta 1	NC	NC	2.1	NC	NC	2.1
NM_019886.1 Carbohydrate sulfotransferase 7	NC	NC	NC	1.7	1.4	2
NM_012200.2 Glucuronosyltransferase I	NC	NC	NC	12.6	NC	NC
AB009598 Glucuronyltransferase I	NC	NC	NC	2.3	NC	NC
Cell growth, cell cycle, apoptosis						
K03193.1 Human epidermal growth factor receptor	−1.4	−2.6	−4.3	1	−2.7	−4.1
BF061658 Transforming growth factor	NC	−1.8	−4	−1.2	−5.1	−6.4
NM_003238.1 Transforming growth factor	NC	−2	−6.9	−1.2	−3.5	−4.9
NM_000358.1 Transforming growth factor	−1	−1.1	−2.1	−1	−3.3	−3.7
M19154.1 Human transforming growth factor-β-2	NC	−2	−5.4	−1	−3	−4.7
NM_002006.1 Fibroblast growth factor 2	NC	−1.9	−4.9	NC	−1.8	−1.3
AI434345 Activating transcription factor 1	−1.2	−1.4	−3.8	NC	−1.8	−2.2
Z24725.1 Mitogen-inducible gene mig-2	NC	−1.4	−2.2	−1.1	−2.4	−2
NM_004702.1 Cyclin E2 (CCNE2)	NC	NC	−1.7	NC	−2.3	−4.3
AF112857.1 Cyclin E2 splice variant 1	NC	NC	−1.3	−1	−2.8	−5.4
NM_021960.1 Myeloid cell leukemia sequence (BCL2-related)	−1.1	NC	−1.5	−1	−2.3	−3.4
NM_004049.1 BCL2-related protein A1 (BCL2A1)	NC	NC	−1.4	−1.2	−2.1	−3.5
D64137 KIP2 Gene for Cdk-inhibitor p57KIP2	NC	2.2	4.3	2.3	4.5	4.5
NM_000076.1 Cyclin-dependent kinase inhibitor 1C(p57Kip2)	NC	1.6	1.1	2.1	3.4	4.7
NM_030579.1 Cytochrome b5	−1.4	−1.4	−6.9	NC	−1.6	−1.9
Cell signal transduction pathways						
NM_003954.1 MAP kinase kinase kinase 14	−1.1	−1.6	−2.3	−1.1	−2.7	−3.9
NM_002756.1 MAP kinase kinase 3 (MAP2K3)	−1.2	1.1	−1.7	NC	−2.3	−2.7
NM_002376.1 MAP regulating kinase 3 (MARK3)	−1.3	−1.7	−3.3	NC	−2.2	−3.1
NM_003010.1 MAP kinase kinase 4 (MAP2K4)	−1.3	−1.7	−4.1	−1.1	−2	−2.9
NM_003618.1 MAP kinase kinase kinase kinase 3 (MAP4K3)	−1.4	−2	−6.8	NC	−2	−2.4
NM_006218.1 Phosphoinositide-3-kinase	−1.4	−1.2	−2.1	NC	NC	NC
NM_002646.1 Phosphoinositide-3-kinase	−3	−1.4	−2.4	NC	NC	NC
Transcription factors, oncogenesis, angiogenesis, other						
NM_007111.1 Transcription factor Dp-1 (TFDP1)	−1.2	−1.3	−3.8	NC	−2.1	−3.7
U62296.1 Transcription factor NF-YC	−1.2	−1.5	−3.4	NC	−2	−2.1
BE966878 Eukaryotic protein synthesis initiation factor 4	NC	NC	NC	−1.1	−1.8	−2.1
NM_001755.1 Core-binding factor, β subunit (CBFB)	−1.2	−1.6	−2.9	NC	−2	−2.1
NM_006850.1 Suppression of tumorigenicity 16 (ST16)	2.2	3.6	2.4	1.2	1.7	2.4
AI812030 Thrombospondin 1	NC	−1.9	−4.7	−1	−3	−4.3
BE999967 Thrombospondin 1	−1	−1.4	−3.9	−1.2	−3.3	−5
BE620457 Neuropilin 1	−1.6	−1.9	−8.2	NC	NC	NC
M92934.1 Connective tissue growth factor	NC	NC	NC	−1.9	−14.7	−19.8

1 Positive values represent increases, negative values are decreases.
2 Abbreviations: Cdk, cyclin-dependent kinase; MAP, mitogen-activated protein; NF, nuclear factor.
3 No change.

involved in the physiologic processes of prostate cancer cells.

DISCUSSION

Our results from cDNA microarray provided a genome-wide analysis of the cellular response to I3C and DIM treatments. Cellular responses to any antiproliferative agents involve modulation of complex pathways that ultimately determine whether a cell survives or dies. Our data revealed that the alterations of biological processes and molecular functions in I3C- and DIM-treated PC3 cells are complex and are likely to be mediated by a variety of regulatory pathways. First, we found that I3C and DIM regulated the expression of phase I and II enzymes in PC3 prostate cancer cells, suggesting the anticarcinogenic effects of I3C and DIM. Carcinogenesis is a multistage process, and there is great opportunity for intervention to stop, revert or delay the carcinogenic process. One

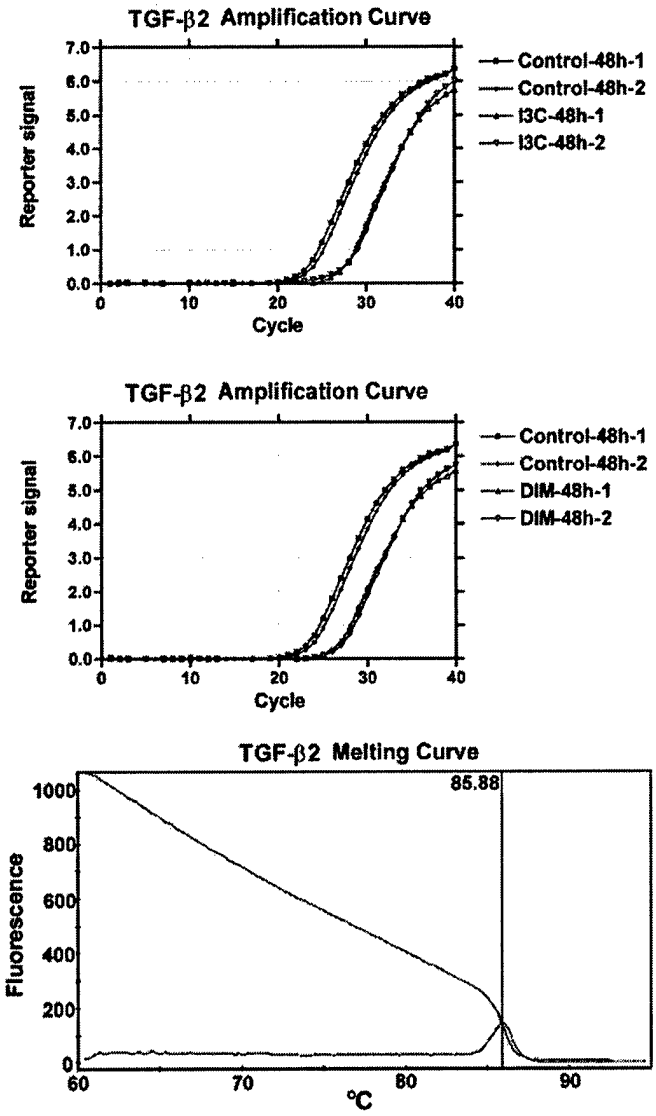

FIGURE 3 Real-time reverse transcription-polymerase chain reaction (RT-PCR) amplification curves showing the amplification of transforming growth factor (TGF)-β2 from RNA of PC3 cells treated with 60 μmol/L indole-3-carbinol (I3C) (*upper panel*) or 40 μmol/L 3,3'-diindolylmethane (DIM) (*middle panel*) for 48h. 48h-1 and 48h-2 are duplicated experiments for 48h treatment sample. *Lower panel*: the real-time RT-PCR melting curve showing the product is pure (only one peak).

anticarcinogenic action is the modulation of metabolism of carcinogens, including inhibition of procarcinogen activation, induction of detoxification and blocking of reactive metabolites (3). The molecules involved in this modulation are phase I and phase II biotransformation enzymes. The most important phase I enzymes are the cytochrome P_{450} (CYP) enzymes. These enzymes oxidize carcinogens and make them more hydrophilic as well as susceptible to detoxification (3,26). Phase II metabolism comprises detoxification and conjugation reactions, making phase I metabolites more polar and readily excretable (3,26). We found that I3C and DIM up-regulated the expression of the phase I enzyme CYP1A1 and the phase II enzymes, glutathione S-transferase theta 1 and aldo-keto reductase, in PC3 prostate cancer cells, suggesting their increased capacity for detoxification and inhibition of carcinogens. It has been reported that both I3C and DIM elevated the activity of phase I and phase II enzymes in rat liver (3). Therefore, our microarray data are consistent with the in vivo experimental reports published by other investigators and provide molecular evidence that I3C and DIM may serve as chemopreventive agents against prostate cancer because of their ability to induce phase I and phase II enzymes.

Because DIM is one of the major in vivo dimeric products of I3C, we also found that the molecular response to both I3C and DIM in PC3 prostate cancer cells involved mainly inhibition of genes that are related to cell growth, cell cycle control, apoptosis, signal transduction, oncogenesis, transcription regulation and protein phosphorylation in addition to the induction of phase I and II enzymes. Epidermal growth factor receptor (EGFR) is a cell membrane protein that is overexpressed in many cancers that have a poor prognosis. This cell membrane protein has been considered to be an excellent target for antitumor therapy (27). TGF-β and fibroblast growth factor (FGF) are multifunctional and essential to the survival of cancer cells. They play important roles in promoting cell growth and angiogenesis (28,29). We observed down-regulation of the EGFR, TGFβ2 and FGF by I3C and DIM treatment, demonstrating the inhibitory effects of I3C and DIM on the growth of PC3 prostate cancer cells. Cyclin E, activating transcription factor (ATF) and mitogen-inducible gene (MIG) regulate cell cycle progression, and BCL2 inhibits apoptotic cell death (30–33). The overexpression of ATF has also been observed in metastatic cancer cells (34). $p57^{KIP2}$ is a tumor suppressor that inhibits cyclin-dependent kinase and results in cell cycle arrest (35). Our results showed that I3C and DIM inhibited the expression of cyclin E2, ATF5, MIG-2 and BCL2, and induced the expression of $p57^{KIP2}$, which may lead to the induction of cell cycle arrest and apoptosis. Taken together, I3C and DIM appear to modulate the expression of several genes that may contribute to the observed cell growth inhibition as demonstrated by the MTT cell growth inhibition assay.

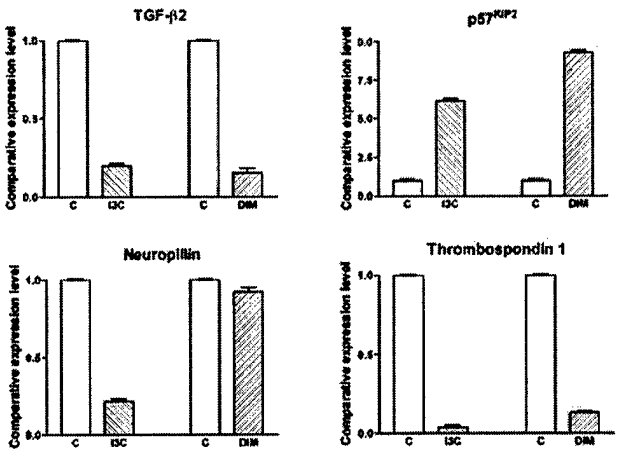

FIGURE 4 Real-time reverse transcription-polymerase chain reaction analysis of selected genes showing alterations in mRNA expression of the specific genes in PC3 cells treated with 60 μmol/L indole-3-carbinol (I3C) or 40 μmol/L 3,3'-diindolylmethane (DIM) for 48 h compared with control PC3 cells. C represents the solvent control; TGF, transforming growth factor. Data are means ± SEM, $n = 3$; calculated by the comparative cycle threshold method.

Cell signal transduction pathways are important for cell survival. Recently, the MAPK and phosphatidylinositol-3-kinase (PI3K)/Akt pathways have received much attention in cancer research and are believed to be excellent targets for cancer prevention and therapy. The MAPK pathway consists of a three-tiered kinase core in which a MAP3K activates a MAP2K that activates a MAPK, resulting in the activation of NF-κB, cell growth and cell survival (36,37). We observed down-regulation in the expression of MAP2K3, MAP2K4, MAP4K3, and MARK3 by I3C and DIM treatments, suggesting that I3C and DIM have inhibitory effects on the MAPK pathway, resulting in the abrogation of cancer cell survival. PI3K/Akt pathway is another important signal transduction pathway and plays a critical role in controlling cell survival and apoptosis (38). From the gene expression profiles of PC3 cells exposed to I3C, we found down-regulation of PI3K expression, suggesting that I3C could induce apoptosis and inhibit cancer cell survival by altering the PI3K/Akt pathway. These findings from cDNA microarray gene analysis are consistent with our previous report showing that I3C inhibits cancer cell growth and induces apoptosis by inhibiting the NF-κB and Akt signal pathways (21,22).

Several Pol II transcription factors, including transcription factor Dp-1 (TFDP) and NF-YC, play important roles in cell transcription (39,40). TFDP1 overexpression leads to up-regulation of cyclin E, which encodes a positive regulator for cell cycle G_1/S transition (39). The overexpression of these transcription factors has also been related to oncogenesis. In addition, core binding factor β (CBFβ) and suppressor of tumorigenicity 16 (ST16) are also involved in oncogenesis. CBFβ forms a fusion protein with other gene products and promotes oncogenesis (41), whereas ST16 suppresses oncogenesis (42). Our results showed that I3C and DIM down-regulated the expression of TFDP1, NF-YC, DKC1, cyclin E and CBFB, and up-regulated ST16 expression, suggesting that I3C and DIM can inhibit transcription and oncogenesis, and could also induce G_1 arrest, as demonstrated by our previous study (22).

It is important to note, however, that I3C and DIM also had some differential effects on the gene expression profile, which is perhaps expected. We observed that DIM but not I3C induced the expression of other phase II enzymes including methylmalonate-semialdehyde dehydrogenase, phospholipase A2, carbohydrate sulfotransferase 7 and glucuronosyltransferase I, suggesting that DIM may have a more inhibitory effect on oncogenesis than I3C.

In summary, the goal of the present study was to analyze the gene expression profiles of PC3 prostate cancer cells after exposing them to I3C and DIM. Both I3C and DIM changed the expression of a large number of genes that are related to the control of carcinogenesis, cell survival and physiologic behaviors. This may help determine the molecular mechanism(s) by which I3C and DIM exert their pleiotropic effects on PC3 prostate cancer cells; such information could be further exploited for devising chemopreventive and/or therapeutic strategies for prostate cancer.

LITERATURE CITED

1. Heber, D. & Bowerman, S. (2001) Applying science to changing dietary patterns. J. Nutr. 131: 3078S–3081S.

2. Bal, D. G., Foerster, S. B., Backman, D. R. & Lyman, D. O. (2001) Dietary change and cancer: challenges and future direction. J. Nutr. 131: 181S–185S.

3. Verhoeven, D. T., Verhagen, H., Goldbohm, R. A., van den Brandt, P. A. & van Poppel, G. (1997) A review of mechanisms underlying anticarcinogenicity by Brassica vegetables. Chem. Biol. Interact. 103: 79–129.

4. Verhoeven, D. T., Goldbohm, R. A., van Poppel, G., Verhagen, H. & van den Brandt, P. A. (1996) Epidemiological studies on Brassica vegetables and cancer risk. Cancer Epidemiol. Biomark. Prev. 5: 733–748.

5. Bradlow, H. L., Sepkovic, D. W., Telang, N. T. & Osborne, M. P. (1995) Indole-3-carbinol. A novel approach to breast cancer prevention. Ann. N.Y. Acad. Sci. 768: 180–200.

6. Brignall, M. S. (2001) Prevention and treatment of cancer with indole-3-carbinol. Altern. Med. Rev. 6: 580–589.

7. Auborn, K., Abramson, A., Bradlow, H. L., Sepkovic, D. & Mullooly, V. (1998) Estrogen metabolism and laryngeal papillomatosis: a pilot study on dietary prevention. Anticancer Res. 18: 4569–4573.

8. Rosen, C. A., Woodson, G. E., Thompson, J. W., Hengesteg, A. P. & Bradlow, H. L. (1998) Preliminary results of the use of indole-3-carbinol for recurrent respiratory papillomatosis. Otolaryngol. Head Neck Surg. 118: 810–815.

9. He, Y. H., Smale, M. H. & Schut, H. A. (1997) Chemopreventive properties of indole-3-carbinol (I3C): inhibition of DNA adduct formation of the dietary carcinogen, 2-amino-1-methyl-6-phenylimidazo [4, 5-b]pyridine (PhIP), in female F344 rats. J. Cell. Biochem. Suppl. 27: 42–51.

10. Cover, C. M., Hsieh, S. J., Tran, S. H., Hallden, G., Kim, G. S., Bjeldanes, L. F. & Firestone, G. L. (1998) Indole-3-carbinol inhibits the expression of cyclin-dependent kinase-6 and induces a G1 cell cycle arrest of human breast cancer cells independent of estrogen receptor signaling. J. Biol. Chem. 273: 3838–3847.

11. Hong, C., Kim, H. A., Firestone, G. L. & Bjeldanes, L. F. (2002) 3, 3'-Diindolylmethane (DIM) induces a G(1) cell cycle arrest in human breast cancer cells that is accompanied by Sp1-mediated activation of p21(WAF1/CIP1) expression. Carcinogenesis 23: 1297–1305.

12. Ge, X., Yannai, S., Rennert, G., Gruener, N. & Fares, F. A. (1996) 3, 3'-Diindolylmethane induces apoptosis in human cancer cells. Biochem. Biophys. Res. Commun. 228: 153–158.

13. Riby, J. E., Chang, G. H., Firestone, G. L. & Bjeldanes, L. F. (2000) Ligand-independent activation of estrogen receptor function by 3, 3'-diindolylmethane in human breast cancer cells. Biochem. Pharmacol. 60: 167–177.

14. American Cancer Society (2002) Cancer Facts & Figures 2002, pp. 5–6. American Cancer Society, Atlanta, GA.

15. Gopalkrishnan, R. V., Kang, D. C. & Fisher, P. B. (2001) Molecular markers and determinants of prostate cancer metastasis. J. Cell. Physiol. 189: 245–256.

16. Brown, P. O. & Botstein, D. (1999) Exploring the new world of the genome with DNA microarrays. Nat. Genet. 21: 33–37.

17. Xie, W., Mertens, J. C., Reiss, D. J., Rimm, D. L., Camp, R. L., Haffty, B. G. & Reiss, M. (2002) Alterations of Smad signaling in human breast carcinoma are associated with poor outcome: a tissue microarray study. Cancer Res. 62: 497–505.

18. Xu, J., Stolk, J. A., Zhang, X., Silva, S. J., Houghton, R. L., Matsumura, M., Vedvick, T. S., Leslie, K. B., Badaro, R. & Reed, S. G. (2000) Identification of differentially expressed genes in human prostate cancer using subtraction and microarray. Cancer Res. 60: 1677–1682.

19. Kudoh, K., Ramanna, M., Ravatn, R., Elkahloun, A. G., Bittner, M. L., Meltzer, P. S., Trent, J. M., Dalton, W. S. & Chin, K. V. (2000) Monitoring the expression profiles of doxorubicin-induced and doxorubicin-resistant cancer cells by cDNA microarray. Cancer Res. 60: 4161–4166.

20. Zembutsu, H., Ohnishi, Y., Tsunoda, T., Furukawa, Y., Katagiri, T., Ueyama, Y., Tamaoki, N., Nomura, T., Kitahara, O., Yanagawa, R., Hirata, K. & Nakamura, Y. (2002) Genome-wide cDNA microarray screening to correlate gene expression profiles with sensitivity of 85 human cancer xenografts to anticancer drugs. Cancer Res. 62: 518–527.

21. Chinni, S. R. & Sarkar, F. H. (2002) Akt inactivation is a key event in indole-3-carbinol-induced apoptosis in PC-3 cells. Clin. Cancer Res. 8: 1228–1236.

22. Chinni, S. R., Li, Y., Upadhyay, S., Koppolu, P. K. & Sarkar, F. H. (2001) Indole-3-carbinol (I3C) induced cell growth inhibition, G1 cell cycle arrest and apoptosis in prostate cancer cells. Oncogene 20: 2927–2936.

23. Eisen, M. B., Spellman, P. T., Brown, P. O. & Botstein, D. (1998) Cluster analysis and display of genome-wide expression patterns. Proc. Natl. Acad. Sci. U.S.A 95: 14863–14868.

24. Khatri, P., Draghici, S., Ostermeier, G. C. & Krawetz, S. A. (2002) Profiling gene expression using onto-express. Genomics 79: 266–270.

25. Li, Y. & Sarkar, F. (2002) Down-regulation of invasion and angiogenesis-related genes identified by cDNA microarray analysis of PC3 prostate cancer cells treated with genistein. Cancer Lett. 186: 157.

26. van Iersel, M. L., Verhagen, H. & van Bladeren, P. J. (1999) The role of biotransformation in dietary (anti)carcinogenesis. Mutat. Res. 443: 259–270.

27. Mendelsohn, J. (2002) Targeting the epidermal growth factor receptor for cancer therapy. J. Clin. Oncol. 20: 1S–13S.

28. Platten, M., Wick, W. & Weller, M. (2001) Malignant glioma biology: role for TGF-beta in growth, motility, angiogenesis, and immune escape. Microsc. Res. Tech. 52: 401–410.

29. Cross, M. J. & Claesson-Welsh, L. (2001) FGF and VEGF function in angiogenesis: signalling pathways, biological responses and therapeutic inhibition. Trends Pharmacol. Sci. 22: 201–207.

30. Schneider, G., Oswald, F., Wahl, C., Greten, F. R., Adler, G. & Schmid, R. M. (2002) Cyclosporine inhibits growth through the activating transcription

factor/cAMP-responsive element-binding protein binding site in the cyclin D1 promoter. J. Biol. Chem. 277: 43599–43607.

31. Wick, M., Burger, C., Brusselbach, S., Lucibello, F. C. & Muller, R. (1994) Identification of serum-inducible genes: different patterns of gene regulation during G0→S and G1→S progression. J. Cell Sci. 107: 227–239.

32. Nakayama, K. I., Hatakeyama, S. & Nakayama, K. (2001) Regulation of the cell cycle at the G1-S transition by proteolysis of cyclin E and p27Kip1. Biochem. Biophys. Res. Commun. 282: 853–860.

33. Cory, S. & Adams, J. M. (2002) The Bcl2 family: regulators of the cellular life-or-death switch. Nat. Rev. Cancer 2: 647–656.

34. Jean, D. & Bar-Eli, M. (2001) Targeting the ATF-1/CREB transcription factors by single chain Fv fragment in human melanoma: potential modality for cancer therapy. Crit. Rev. Immunol. 21: 275–286.

35. Lee, M. H. & Yang, H. Y. (2001) Negative regulators of cyclin-dependent kinases and their roles in cancers. Cell Mol. Life Sci. 58: 1907–1922.

36. Seger, R. & Krebs, E. G. (1995) The MAPK signaling cascade. FASEB J. 9: 726–735.

37. Sebolt-Leopold, J. S. (2000) Development of anticancer drugs targeting the MAP kinase pathway. Oncogene 19: 6594–6599.

38. Cantley, L. C. (2002) The phosphoinositide 3-kinase pathway. Science (Washington, DC) 296: 1655–1657.

39. Yasui, K., Arii, S., Zhao, C., Imoto, I., Ueda, M., Nagai, H., Emi, M. & Inazawa, J. (2002) TFDP1, CUL4A, and CDC16 identified as targets for amplification at 13q34 in hepatocellular carcinomas. Hepatology 35: 1476–1484.

40. Izumi, H., Molander, C., Penn, L. Z., Ishisaki, A., Kohno, K. & Funa, K. (2001) Mechanism for the transcriptional repression by c-Myc on PDGF beta-receptor. J. Cell Sci. 114: 1533–1544.

41. Kundu, M. & Liu, P. P. (2001) Function of the inv(16) fusion gene CBFB-MYH11. Curr. Opin. Hematol. 8: 201–205.

42. Jiang, H., Su, Z. Z., Lin, J. J., Goldstein, N. I., Young, C. S. & Fisher, P. B. (1996) The melanoma differentiation associated gene mda-7 suppresses cancer cell growth. Proc. Natl. Acad. Sci. U.S.A. 93: 9160–9165.

#6551 Gene expression profiles of I3C and DIM treated PC3 prostate cancer cells by cDNA microarray analysis. Yiwei Li, Xingli Li, and Fazlul H. Sarkar. *Wayne State University School of Medicine, Detroit, MI.*

Studies from our laboratory and others have shown that Indole-3-carbinol (I3C) and its in vivo dimeric product 3,3-diindolylmethane (DIM) inhibit the growth of PC3 prostate cancer cells and induce apoptosis by inhibiting NF-kB and Akt pathways. In order to obtain a comprehensive gene expression profiles altered by I3C and DIM treatment of PC3 cells, we have utilized cDNA microarray to interrogate 22,215 known genes by using Affymetrix Human Genome U133A Array. The total RNA from PC3 cells untreated and treated with 60μM I3C or 40μM DIM for 6 to 48 hours was subjected to microarray analysis and the data were analyzed using Microarray Suite and Data Mining, Cluster and TreeView, and Onto-express software. We found a total of 738 genes which showed >2 fold change after 24 hours of DIM treatment. Among these genes, 677 genes were down regulated and 61 genes were up-regulated. Similarly, 727 genes showed >2 fold change in the expression with down-regulation of 685 genes and up-regulation of 42 genes in I3C treated cells. The altered expressions of genes were observed as early as 6 hours and were significantly more evident with longer treatment. Upon cluster analysis, we found that both I3C and DIM up-regulated expression of genes that are related to the Phase I and Phase II enzymes (p4501A1, GSTT1, etc), suggesting their increased capacity for detoxification of carcinogens or chemicals. We also found that I3C and DIM down regulated the expression of genes, which are critically involved in the regulation of cell growth (EGFR, TGFβ2, IGFBP4, FGF, etc), cell cycle (cyclin E2, ATF5, Mig-2, etc), apoptosis (BCL2A1, etc), signal transduction (PI3K, MAPK, MAP2K3, MAP2K4, MAP4K3, etc), Pol II transcription factor (TFDP1, NF-YC, DKC1, etc), and oncogenesis (CBFB, ST16, etc). However, I3C and DIM also showed some differential effect on gene expression profile, which will be the subject of further study in our laboratory. In order to confirm our microarray data, we have also conducted Real-time RT-PCR analysis of selected genes. The results of Real-time RT-PCR and cDNA microarray data showed highest concordance. From these results, we conclude that I3C and DIM caused changes in the expression of a large number of genes that are related to the control of carcinogenesis, cell survival, and physiological behaviors. This may provide molecular mechanism(s) by which I3C and DIM exert their pleiotropic effects on PC3 prostate cancer cells and these information could be further exploited for devising chemopreventive and/or therapeutic strategies for prostate cancer.

REVIEW

Significance of indole-3-carbinol and its metabolite in human cancers

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Abstract: Epidemiological studies have demonstrated that a significant difference exists in the incidence of cancer among different ethnic groups. This difference is known to be partly attributed to dietary factors. Indole-3-carbinol (I3C) and its in vivo dimeric product 3,3′-diindolylmethane (DIM) are produced from naturally occurring glucosinolates in the family Cruciferae. They have received much attention as dietary components that have an inhibitory affect on cancer. I3C and DIM up-regulate the expression of phase I and phase II enzymes, suggesting an increased role in the detoxification and inhibition of carcinogens. They also inhibit the growth of cancer cells through the modulation of genes that are related to the control of cell proliferation, cell cycle, apoptosis, signal transduction, oncogenesis, transcription regulation and protein phosphorylation. Moreover, I3C and DIM inactivate NF-κB, Akt and MAPK signalling pathways, all of which are believed to be potential targets in cancer prevention and therapy. Collectively, several studies have provided evidence for pre-clinical and clinical activities of I3C and DIM against some cancers. Hence, significant advances have been made to date that show I3C and DIM as promising agents for cancer chemoprevention and/or treatment. This review summarises the well accepted inhibitory effects of I3C and DIM on cancer cells and provides a comprehensive view on their molecular mechanism(s) of cancer chemoprevention.

Keywords: I3C, DIM, cancer, prevention, treatment

Introduction

Epidemiological studies have demonstrated that a significant difference exists in the incidence of cancer among different ethnic groups. This difference is known to be partly attributed to dietary factors. Studies from in vitro and in vivo experiments have shown that dietary factors regulate the processes of carcinogenesis, including the initiation, promotion and progression of human cancers (Meyskens 1992; Yang et al 2001). It has been reported that certain components of plant foods, such as fiber, isoflavone and hydrolysis products of glucosinolates, may be protective against cancers (Adlercreutz et al 1995; Steinmetz and Potter 1996; Verhoeven et al 1997). Indole-3-carbinol (I3C) is produced from naturally occurring glucosinolates, contained in a wide variety of plants including members of the family Cruciferae and, in particular, members of the genus *Brassica* (Broadbent TA and Broadbent HS 1998). I3C is biologically active, and is easily converted in vivo to its dimeric product 3,3′-diindolylmethane (DIM), which is also biologically active. Over one hundred glucosinolates have been identified, predominantly in vegetables of the family Cruciferae (Verhoeven et al 1997; Johnson 2002). In this family, vegetables of the genus *Brassica* contribute most to our intake of glucosinolates, and include all kinds of cabbages, broccoli, cauliflower and Brussels sprouts.

All glucosinolates share a common basic skeleton containing a glucose group, a side chain and a sulphonated oxime moiety, but differ in side chain R (Figure 1). Glucosinolate hydrolysis products make a significant contribution to the health benefit of brassicaceous vegetables (Tawfiq et al 1995; Johnson 2002). The enzyme myrosinase, which is found in plant cells and also in certain intestinal microflora, catalyses the hydrolysis of glucosinolates (Verkerk et al 1997; Johnson 2002). The glucosinolate hydrolysis products include equimolar amounts of aglucon, glucose and sulphate. The aglucones are unstable and undergo further reactions. The nature of aglucones depends primarily on the side chain of the glucosinolate (Tawfiq et al 1995; Verhoeven et al 1997). Glucosinolates with an indole side chain form indoles. The

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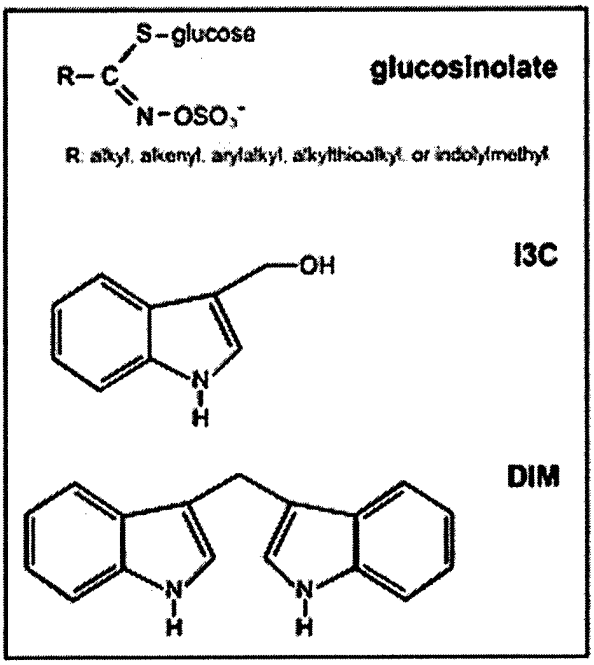

Figure 1 Molecular structure of glucosinolate.

most prevalent glucosinolate with an indole side chain is glucobrassicin, which is predominant in brassicaceous vegetables. When hydrolysis occurs, glucobrassicin forms an unstable isothiocyanate, which degrades to I3C (Verhoeven et al 1997; Broadbent TA and Broadbent HS 1998). I3C is the immediate precursor of DIM. Under the acidic conditions of the stomach, I3C undergoes extensive and rapid self-condensation reactions to form several derivatives (Dashwood et al 1994; Verhoeven et al 1997). DIM is the major derivative and condensation product of I3C (Figure 1), and its formation from I3C has been believed to be a likely prerequisite for I3C-induced anticarcinogenesis (Dashwood et al 1994).

It has been reported that I3C and DIM possess anticarcinogenic effects in experimental animals and inhibit the growth of human cancer cells (He et al 1997; Oganesian et al 1997; Cover et al 1998; Katdare et al 1998; Jin et al 1999; Chen DZ et al 2001; Murillo and Mehta 2001; Hong, Firestone et al 2002; Hong, Kim et al 2002). I3C has also been found to sensitise multidrug resistance (MDR) tumours to chemotherapeutic drugs without eliciting direct toxicity to the host (Christensen and LeBlanc 1996). Moreover, I3C may inhibit breast cancer invasion and migration (Meng, Goldberg et al 2000; Meng, Qi et al 2000). Because of these pleiotropic effects, the interest in I3C and DIM as cancer chemopreventive agents has increased significantly in the past years. Although dietary, epidemiological and experimental studies have shown the benefits of I3C and DIM in the prevention and inhibition of cancer, the molecular mechanism(s) by which I3C and DIM exert their tumour-suppressive effects on cancers have not been fully elucidated. This review summarises the well accepted inhibitory effects of I3C and DIM on cancer cells, and provides a comprehensive view on the molecular mechanism(s) of cancer chemopreventive and therapeutic effects of I3C and DIM.

Inhibition of oncogenesis

Oncogenesis is a multistep process, and there is a great opportunity for intervention to stop, revert or delay the oncogenic process. One anti-oncogenic action is the modulation of carcinogen metabolism, including inhibition of procarcinogen activation, induction of detoxification and blocking of reactive metabolites (Verhoeven et al 1997; van Iersel et al 1999). The molecules involved in this modulation are phase I and phase II biotransformation enzymes. I3C has been shown to inhibit chemically induced tumourigenesis of the liver, mammary gland, colon and other tissues, and suppress spontaneous carcinogenesis in mammary gland and endometrium (Bradlow et al 1991; Kojima et al 1994; Verhoeven et al 1997). It has been reported that treatment of rat with I3C increases CYP1A1, 1A2, 2B and 3A activity in rat liver (Stresser et al 1994; Manson et al 1998). I3C also up-regulates the level and activity of glutathione S-transferases (GST) (Bradfield and Bjeldanes 1984; Manson et al 1998). Therefore, the anti-oncogenic activity of I3C administered before or concurrently with a carcinogen is thought to be mediated through alternations in the levels and activities of phase I (eg p450 or CYP) and phase II (eg GST) isozymes in the liver and/or extrahepatic tissues, resulting in their increased capacity for detoxification of carcinogens.

The authors utilised cDNA microarray technique to determine the alternation of gene expression profiles of PC3 prostate cancer cells exposed to I3C and DIM. From microarray analysis, both I3C and DIM up-regulated the expression of phase I and II enzymes in PC3 prostate cancer cells (Li et al 2003). The most important phase I enzymes are cytochrome p450 enzymes, which oxidise carcinogens and make them more hydrophilic and susceptible to detoxification. Phase II metabolism comprises detoxification and conjugation reactions, making phase I metabolites more polar and readily excretable. I3C and DIM up-regulated

the expression of phase I enzyme cytochrome p450 1A1 (CYP1A1), phase II enzymes glutathione S-transferase theta 1 (GSTT1) and aldo-keto reductase (AKR) in PC3 prostate cancer cells, suggesting their increased capacity for detoxification and inhibition of carcinogens (Li et al 2003). These results are consistent with the experimental reports published by other investigators (Bradfield and Bjeldanes 1984; Vang et al 1990; Wortelboer et al 1992; Takahashi et al 1995; Lake et al 1998; Manson et al 1998; Katchamart and Williams 2001; Nho and Jeffery 2001; Horn et al 2002). They provide molecular evidence demonstrating that I3C and DIM may serve as a chemopreventive agent against prostate and other human cancers because of their ability to induce phase I and phase II enzymes.

From the cDNA microarray analysis, it was also found that DIM, but not I3C, induced the expression of other phase II enzymes, including methylmalonate-semialdehyde dehydrogenase, phospholipase A2, carbohydrate sulfotransferase 7 and glucuronosyltransferase I, indicating that DIM may have more of an inhibitory effect on oncogenesis compared to I3C (Li et al 2003). These results are very interesting and require further in-depth investigation (currently in progress).

It has been well known that oestrogen and androgen elicit their oncogenic actions in hormone-related cancers, such as breast, cervical, endometrium and prostate cancers. I3C has been shown to suppress tumourigenesis of oestrogen-responsive tissues (Bradlow et al 1991; Kojima et al 1994). Clinical trials also show that I3C at a minimum effective dose schedule is a promising chemopreventive agent for breast cancer prevention (Wong et al 1997). Studies from several laboratories have shown that I3C is a negative regulator of the oestrogen receptor (OR) signalling pathway (Riby et al 2000; Auborn et al 2003). By competitive binding to OR, I3C was found to inhibit the expression of genes driven by OR, resulting in cell growth inhibition and apoptosis. In addition, I3C has been shown to induce oestradiol metabolism through the induction of cytochrome p450 (CYP), resulting in a decrease in active oestrogen (Horn et al 2002). Clinical trials also show that I3C could be useful to increase the 2-OH-oestrone:oestriol metabolite ratio without detectable side effects in women (Bradlow et al 1994). Moreover, DIM binds to AhR (aryl hydrocarbon receptor), functions as an AhR agonist and down-regulates oestrogen-induced genes by blocking the crosstalk between AhR and OR (Chen I et al 1996; Chen et al 1998; Chen I et al 2001). A recent study shows that DIM is a strong androgen antagonist that blocks expression of androgen-responsive genes and inhibits androgen receptor nuclear translocation. This results in the inhibition of cell proliferation and DNA synthesis in LNCaP prostate cancer cells (Le et al 2003). These results demonstrate that I3C and DIM also exert their anti-oncogenic effects through modulation of OR, AR and AhR signalling pathways. However, it is important to note that I3C and DIM show inhibitory effects on both hormone-related and unrelated cancers, suggesting the comprehensive molecular mechanisms of their actions. Hence, the focus of this review is primarily based on recent molecular knowledge that may be applicable to cancer cells irrespective of their status of OR, AR, AhR or cytochrome p450.

Inhibition of cancer cell growth and induction of cell cycle arrest

It has been shown that I3C and DIM inhibit the growth of cancer cells including breast, prostate, colon and cervical cancer cells (Jin et al 1999; Bonnesen et al 2001; Chen DZ et al 2001; Hong, Firestone et al 2002; Hong, Kim et al 2002; Frydoonfar et al 2003; Kim et al 2003; Nachshon-Kedmi et al 2003). The authors have experimentally studied the effects of I3C and DIM on the growth of various cancer cells, including PC3 prostate cancer cells, MDA-MB-231, MDA-MB-435, and MCF10CA1a breast cancer cells (Rahman et al 2000; Chinni et al 2001; Li et al 2003; Rahman et al 2003). The growth of these cancer cells was inhibited by treatment with 30–100 μM I3C or 20–60 μM DIM for a period of 24–72 hours. The inhibition of cell growth was found to be dose- and time-dependent. These effects of I3C and DIM clearly indicate that they could be very useful for inhibiting the cell growth of various cancer cells.

The growth inhibition of cancer cells could be due to cell-cycle arrest, which ultimately results in ceasing cell proliferation. It has been reported that I3C can induce a G1 cell cycle arrest in breast cancer cells (Cover et al 1998; Hong, Kim et al 2002). Data also showed that I3C induced a G1 cell cycle arrest in PC3 prostate cancer cells (Chinni et al 2001). The induction of cell cycle arrest in breast and prostate cancer cells by I3C could be mediated through the regulation of expression of genes involved in the control of the cell cycle.

The progression of the cell cycle is known to be tightly regulated by different cyclins, cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CDKIs)

in different phases of the cell cycle. The data showed a dose- and time-dependent decrease in the expression of CDK6 in I3C-treated PC3 prostate cancer cells (Chinni et al 2001). Moreover, I3C inhibited the CDK6 kinase activity, which plays important roles in the regulation of G1 phase progression (Chinni et al 2001). Other studies have also reported that I3C inhibited the expression of CDK6 in breast cancer cells (Cover et al 1998; Cram et al 2001). Cyclins/CDKs complex is needed for cells passing through the cell cycle; however, the activity of cyclins/CDKs complex is negatively regulated by several CDK inhibitors (CDKIs) including $p21^{WAF1}$, $p27^{KIP1}$ and p16. The authors examined whether I3C could alter the expression of $p21^{WAF1}$ and $p27^{KIP1}$ in PC3 prostate cancer cells by Western blot. The data revealed a significant dose-dependent up-regulation of $p21^{WAF1}$ and $p27^{KIP1}$ expressions in I3C-treated cancer cells compared to control cells (Chinni et al 2001). This finding was consistent with the results on the inhibition of cancer cell growth, cell cycle arrest and down-regulation of CDK6, all of which demonstrate that I3C can inhibit the growth of cancer cells by modulating the expression of genes that are involved in the regulation of cell proliferation and cell cycle. The up-regulation of $p21^{WAF1}$ and $p27^{KIP1}$ and down-regulation of CDK6 may be one of the molecular mechanism(s) by which I3C inhibits cancer cell growth and induces cell cycle arrest (Figure 2).

Induction of apoptosis

In addition to cell cycle arrest, the overall cell growth inhibition induced by I3C could also be due to increased programme cell death known as apoptosis. To address this issue, apoptosis assays were conducted in MDA-MB-435 and MCF10CA1a breast cancer cells, and PC3 prostate cancer cells treated with I3C (Rahman et al 2000; Chinni et al 2001; Rahman et al 2003). For apoptosis assays, nucleosomal DNA ladders are widely used as biochemical markers of apoptosis. The cleavage of poly(ADP-ribose) polymerase (PARP) has also been used as early markers of apoptosis (Lazebnik et al 1994). Additionally, flow cytometric analysis with 7AAD staining has been conducted to detect and quantify apoptotic cells (Philpott et al 1996). By using these techniques, induced apoptosis in all cancer cells tested occured when they were treated with 60–100 μM of I3C. DNA ladder formation and PARP cleavage were observed in cancer cells treated with I3C for 48 hours (Rahman et al 2000; Chinni et al 2001; Rahman et al 2003). Flow cytometry revealed that the number of apoptotic cells increased up to 58%–84% with longer I3C treatment (Rahman et al 2000; Chinni et al 2001; Rahman et al 2003). These results clearly suggest that I3C can induce apoptosis, and are consistent with studies reported by other investigators (Ge et al 1996; Katdare et al 1998; Ge et al 1999; Bonnesen et al 2001; Chen DZ et al 2001; Hong, Firestone et al 2002; Zheng et al 2002; Nachshon-Kedmi et al 2003).

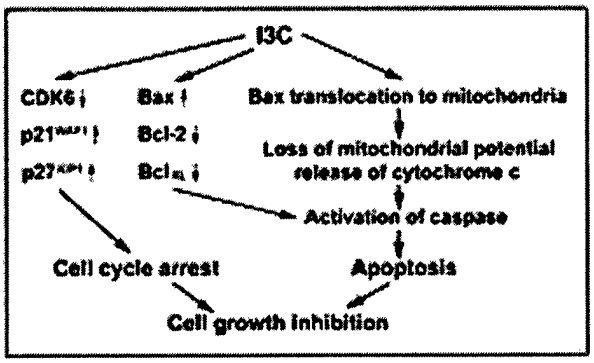

Figure 2 The effects of I3C on cell cycle arrest and apoptosis.

To exploit the molecular mechanism(s) of I3C-induced apoptosis, the authors investigated the alternation of gene expression involved in the apoptotic pathway. Bax, Bcl-2 and Bcl_{XL} have been reported to play a major role in determining whether cells will undergo apoptosis under experimental conditions that promote cell death (Kane et al 1993; Park and Hockenbery 1996; Findley et al 1997; Cory and Adams 2002). Increased expression of Bax could induce apoptosis, while Bcl-2 and Bcl_{XL} protect cells from apoptosis. It has also been found that the ratio of Bax:Bcl-2, rather than Bcl-2 alone, is important for the survival of drug-induced apoptosis (Salomons et al 1997). The data showed a reduced level in Bcl-2 and Bcl_{XL} protein expression in prostate and breast cancer cells tested after treatment with I3C for 48 hours and longer (Rahman et al 2000; Chinni et al 2001; Rahman et al 2003). The expression of Bax was up-regulated after I3C treatment for 24 hours. The ratio of Bax to Bcl-2 was significantly increased after 24 hours of treatment, corresponding with a significant increase in apoptotic cells after 48 hours of I3C treatment. A similar result was also observed in I3C-treated breast cancer cells by other investigators (Hong, Firestone et al 2002). These results suggest that up-regulation of Bax and down-regulation of Bcl-2 and Bcl_{XL} may be one of the molecular mechanism(s) by which I3C and DIM induces apoptosis (Figure 2).

Bax translocation from cytosol into mitochondria has been known as a critical event that occurs during

apoptotic processes (Bedner et al 2000; Jia et al 2001). By using immunostaining and confocal imaging techniques, the translocation of Bax from cytosol into mitochondria in both MCF10A non-tumourigenic and MCF10CA1a cancer cells treated with I3C (Rahman et al 2003) was observed. However, no significant apoptosis was seen in MCF10A non-tumourigenic cells treated with I3C. It has been reported that the translocation of Bax from cytosol into mitochondria targets the mitochondrial intermembrane contact sites. This causes mitochondrial permeability transition, loss of mitochondrial potential, release of cytochrome c, subsequent activation of caspases and DNA fragmentation resulting in apoptotic cell death (Putcha et al 1999; Gao et al 2001; De Giorgi et al 2002). I3C induces the loss of mitochondrial potential and the release of cytochrome c in MCF10CA1a breast cancer cells but not MCF10A non-tumourigenic cells. This occurs even though Bax was translocated from cytosol into mitochondria upon I3C treatment in both cells (Rahman et al 2003), suggesting that I3C-induced loss of mitochondrial potential is a more important and direct event for the release of cytochrome c and induction of apoptosis in cancer cells (Figure 2). The fact that I3C selectively induces the loss of mitochondrial potential, the release of cytochrome c and apoptosis in MCF10CA1a breast cancer cells rather than MCF10A non-tumourigenic cells, makes I3C an ideal agent for preventive and/or therapeutic purposes against breast and other cancers.

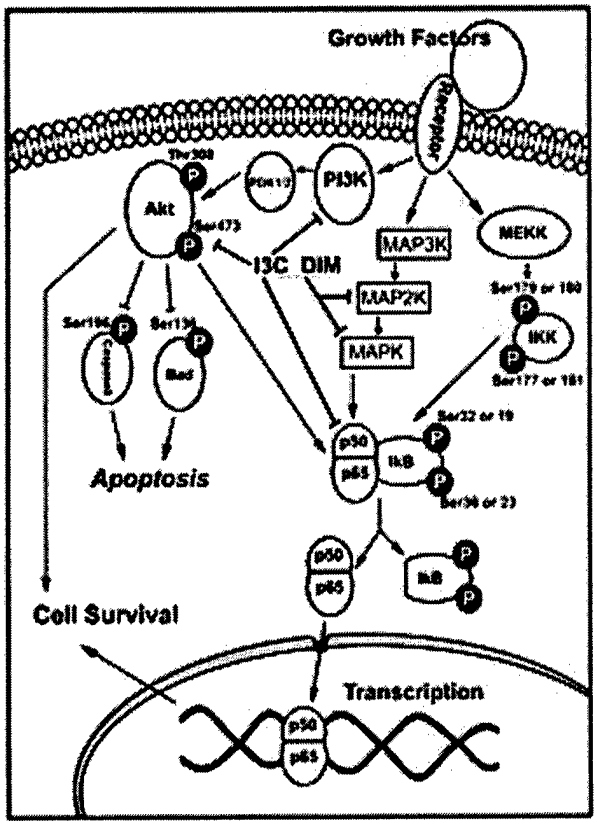

Figure 3 Akt, NF-κB and MAPK signalling pathways and the effects of I3C and DIM on those pathways.

Inhibition of NF-κB and Akt pathways

The nuclear factor-κB (NF-κB) pathway plays an important role in the control of cell growth, differentiation, apoptosis, inflammation, stress response and many other physiological processes in cellular signalling. The NF-κB family is composed of five proteins: RelA (p65), RelB, c-Rel, NF-κB1 (p50) and NF-κB2 (p52), each of which may form homo- or hetero-dimers (Ghosh et al 1995; Muller et al 1995; Verma et al 1995). In human cells, NF-κB is sequestered in the cytoplasm through tight association with its inhibitory protein, IκB. The activation of NF-κB occurs through site-specific phosphorylation and ubiquitination of IκB protein by IKK. IκB is subsequently degraded by the 26S proteasome (Chen et al 1995; Traenckner et al 1995; Chen ZJ et al 1996). This process allows NF-κB to become free from IκB and to translocate into the nucleus for binding to NF-κB-specific DNA-binding sites and, in turn, regulating gene transcription (Figure 3). Recent reports demonstrate that IKK-α also phosphorylates histone H3 and regulates the activation of NF-κB-directed gene expression (Anest et al 2003; Israel 2003; Yamamoto et al 2003). NF-κB controls the expression of many genes that are involved in the cellular and physiological processes as mentioned above. The disorder of these physiological processes has been demonstrated to link with the onset of cancers and chronic diseases. Therefore, NF-κB has been described as a major culprit and a therapeutic target in cancer (Biswas et al 2001; Bharti and Aggarwal 2002; Haefner 2002; Hideshima et al 2002; Orlowski and Baldwin 2002).

Because of the importance of NF-κB, as mentioned above, the authors investigated whether I3C treatment could modulate NF-κB DNA-binding activity in PC3 prostate cancer cells by electrophoresis mobility shift assay (EMSA) (Chinni et al 2001). PC3 cells were treated with 60 μM I3C for 48 hours. Nuclear extracts were harvested from control and I3C-treated cells, incubated in binding buffer with ^{32}P-labelled NF-κB consensus oligonucleotide, and

subjected to 8% non-denatured polyacrymide gel. After drying the gel, autoradiography of the gel revealed that 60 μM I3C significantly inhibited NF-κB DNA binding in PC3 prostate cancer cells.

The Akt signalling pathway is another important signal transduction pathway in human cells. Also referred to as PKB, Akt plays a critical role in controlling the balance between cell survival and apoptosis (Burgering and Coffer 1995). It contains an amino-terminal pleckstrin homology (PH) domain that binds phosphorylated lipids at the membrane in response to activation of PI3 kinases. Akt may be activated by insulin and various growth and survival factors through the activation of PI3 kinase (Franke et al 1995). It is activated by phospholipid binding and phosphorylation at Thr308 by PDK1, and also by phosphorylation within the C-terminus at Ser473 by PDK2 (Alessi et al 1996; Rommel et al 1999). Akt functions to promote cell survival by inhibiting apoptosis through its ability to phosphorylate and inactivate several targets, including Bad, Forkhead transcription factors and caspase-9, all of which are involved in apoptotic pathways (Brunet et al 1999; Rommel et al 1999). Recent reports show that it also regulates the NF-κB pathway via phosphorylation and activation of molecules in the NF-κB pathway (Figure 3) (Ozes et al 1999; Romashkova and Makarov 1999). Akt has also been described as a target in cancer therapy (Chang et al 2003).

Because Akt pathways play an important role in cell growth, apoptosis and oncogenesis, the status of Akt in PC3 cells treated with 30, 60 and 100 μM I3C by immunoprecipitation, Western blot and kinase assays (Chinni and Sarkar 2002) were examined. No alterations on the total Akt protein expression in I3C-treated PC3 cells were found. However, a decrease in the phosphorylated Akt protein at Ser473 and Thr308 was seen in the I3C-treated PC3 cells compared to control cells, suggesting inactivation of Akt kinase after I3C treatment. These results were confirmed by Akt kinase assay, which showed a decrease in the Akt kinase activity in I3C-treated PC3 cells. Examining the Akt status in PC3 cells pretreated with I3C followed by EGF stimulation, it was found that EGF treatment alone activated Akt kinase as expected, while I3C pre-treatment abrogated the activation of Akt by EGF. These data demonstrate that I3C inhibits the activation of Akt and Akt kinase activity, which may result in the inhibition of survival signals and induce apoptotic signals. The inhibition of NF-κB and Akt pathways by I3C may be another molecular mechanism by which I3C induces apoptosis. The fact that I3C inhibits both NF-κB and Akt pathways, which are known as therapeutic targets for cancer therapy, makes I3C an ideal agent for preventive and/or therapeutic purposes against cancer.

Regulation of gene expression profiles

Although interest in the molecular effects of I3C and DIM on cancer cells has greatly increased in recent years, little is known about the global gene expression profiles of cancer cells after I3C or DIM treatment. The precise molecular mechanism(s) by which I3C and DIM exert their tumour suppressive effects is still unclear. To establish such molecular mechanism(s), the high-throughput gene chip, which contains 22 215 known genes, was utilised to determine the alternation of gene expression profiles of PC3 prostate cancer cells exposed to I3C or DIM (Li and Sarkar 2003). The results from cDNA microarrays provided a genome-wide analysis of the cellular response to I3C and DIM treatment. It was found that the alternation of biological processes and molecular functions in I3C- and DIM-treated PC3 cells are complex and are likely to be mediated by a variety of regulatory pathways (Li and Sarkar 2003). Thus, the global gene expression profile of cancer cells after I3C or DIM treatment opens new avenues to further investigate the molecular mechanism(s) by which I3C and DIM inhibit cancer cells.

Through using cDNA microarray analysis, it was found that the molecular response to both I3C and DIM in PC3 prostate cancer cells mainly involves the inhibition of genes that are related to cell proliferation, cell cycle control, apoptosis, signal transduction, oncogenesis and transcription regulation (Li and Sarkar 2003). EGFR, TGF-β2 and FGF play important roles in promoting cell growth and angiogenesis (Cross and Claesson-Welsh 2001; Platten et al 2001; Mendelsohn 2002). The down-regulation of EGFR, TGF-β2 and FGF by I3C or DIM treatment corresponded with the growth inhibitory effects of I3C and DIM. Cyclin E, activating transcription factor (ATF), and mitogen inducible gene (MIG) regulate cell cycle progression and apoptosis (Jean and Bar-Eli 2001; Nakayama et al 2001). The results also showed that I3C and DIM inhibited the expression of cyclin E2, ATF5, MIG-2 and Bcl-2, and induced the expression of $p57^{KIP2}$, which may lead to the induction of cell cycle arrest and apoptosis.

Cell signal transduction pathways are important for cell survival. In addition to NF-κB and Akt pathways targeted by I3C and DIM as mentioned earlier, MAPK has received much attention in cancer research and is believed to be a target for cancer prevention and therapy. The MAPK pathway consists of a three-tiered kinase core where a MAP3K activates a MAP2K that activates a MAPK (Figure 3), resulting in the activation of NF-κB, cell growth and cell survival (Seger and Krebs 1995; Sebolt-Leopold 2000). Down-regulation in the expression of MAP2K3, MAP2K4, MAP4K3 and MAPK3 by I3C and DIM treatment was observed, suggesting the inhibitory effects of I3C and DIM on the MAPK pathway, which is likely to result in the inhibition of cancer cell survival. From the gene expression profiles of PC3 cells exposed to I3C, the down-regulation of PI3K expression was found to be consistent with results showing inactivation of the Akt kinase pathway by I3C.

Several Pol II transcription factors including TFDP and NF-YC play important roles in cell transcription (Yasui et al 2002; Romier et al 2003). TFDP1 overexpression leads to up-regulation of cyclin E, which encodes a positive regulator for cell cycle G1/S transition (Yasui et al 2002). Their overexpression has also been related to oncogenesis. In addition, core binding factor beta (CBF-β) and suppressor of tumourigenicity 16 (ST16) are also known to be involved in the processes of oncogenesis. CBF-β forms fusion protein with other gene product and promotes oncogenesis, while ST16 suppresses the oncogenesis (Jiang et al 1996; Kundu and Liu 2001). The results from gene expression profiling showed that I3C and DIM down-regulated the expression of TFDP1, NF-YC, cyclin E and CBF-β, and up-regulated ST16 expression. This suggests that I3C and DIM-mediated inhibition in transcription and oncogenesis involves multiple genes, all of which could be specific targets of I3C and DIM.

Conclusions

The studies presented in this review article clearly indicate that I3C and DIM, generally obtained from natural food sources, play important roles in cancer prevention and may perhaps be useful for treatment. These effects are believed to be mediated through the regulation of cell cycle, cell proliferation, apoptosis, oncogenesis, transcription and cell signal transduction. Therefore, the inactivation of Akt, NF-κB, MAPK and Bcl-2 signalling pathways may represent the molecular mechanism(s) by which I3C and DIM exert their anticancer effects. However, the biggest challenge is to demonstrate whether specific targets are affected by I3C/DIM in vivo. Therefore, further in vitro and in vivo investigations along with clinical trials are needed to fully appreciate the value of I3C and DIM in the fight against human cancers.

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References

Adlercreutz CH, Goldin BR, Gorbach SL et al. 1995. Soybean phytoestrogen intake and cancer risk. *J Nutr*, 125:757S–770S.

Alessi DR, Andjelkovic M, Caudwell B et al. 1996. Mechanism of activation of protein kinase B by insulin and IGF-1. *EMBO J*, 15: 6541–51.

Anest V, Hanson JL, Cogswell PC et al. 2003. A nucleosomal function for IkappaB kinase-alpha in NF-kappaB-dependent gene expression. *Nature*, 423:659–63.

Auborn KJ, Fan S, Rosen EM et al. 2003. Indole-3-carbinol is a negative regulator of estrogen. *J Nutr*, 133:2470S–2475S.

Bedner E, Li X, Kunicki J et al. 2000. Translocation of Bax to mitochondria during apoptosis measured by laser scanning cytometry. *Cytometry*, 41:83–8.

Bharti AC, Aggarwal BB. 2002. Nuclear factor-kappa B and cancer: its role in prevention and therapy. *Biochem Pharmacol*, 64:883–8.

Biswas DK, Dai SC, Cruz A et al. 2001. The nuclear factor kappa B (NF-kappa B): a potential therapeutic target for estrogen receptor negative breast cancers. *Proc Natl Acad Sci USA*, 98:10386–91.

Bonnesen C, Eggleston IM, Hayes JD. 2001. Dietary indoles and isothiocyanates that are generated from cruciferous vegetables can both stimulate apoptosis and confer protection against DNA damage in human colon cell lines. *Cancer Res*, 61:6120–30.

Bradfield CA, Bjeldanes LF. 1984. Effect of dietary indole-3-carbinol on intestinal and hepatic monooxygenase, glutathione S-transferase and epoxide hydrolase activities in the rat. *Food Chem Toxicol*, 22: 977–82.

Bradlow HL, Michnovicz JJ, Halper M et al. 1994. Long-term responses of women to indole-3-carbinol or a high fiber diet. *Cancer Epidemiol Biomarkers Prev*, 3:591–5.

Bradlow HL, Michnovicz J, Telang NT et al. 1991. Effects of dietary indole-3-carbinol on estradiol metabolism and spontaneous mammary tumors in mice. *Carcinogenesis*, 12:1571–4.

Broadbent TA, Broadbent HS. 1998. 1. The chemistry and pharmacology of indole-3-carbinol (indole-3-methanol) and 3-(methoxymethyl)indole. Part II. *Curr Med Chem*, 5:469–91.

Brunet A, Bonni A, Zigmond MJ et al. 1999. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell*, 96:857–68.

Burgering BM, Coffer PJ. 1995. Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature*, 376: 599–602.

Chang F, Lee JT, Navolanic PM et al. 2003. Involvement of PI3K/Akt pathway in cell cycle progression, apoptosis, and neoplastic transformation: a target for cancer chemotherapy. *Leukemia*, 17: 590–603.

Chen DZ, Qi M, Auborn KJ et al. 2001. Indole-3-carbinol and diindolylmethane induce apoptosis of human cervical cancer cells and in murine HPV16-transgenic preneoplastic cervical epithelium. *J Nutr*, 131:3294–302.

Chen I, Hsieh T, Thomas T et al. 2001. Identification of estrogen-induced genes downregulated by AhR agonists in MCF-7 breast cancer cells using suppression subtractive hybridization. *Gene*, 262:207–14.

Chen I, McDougal A, Wang F et al. 1998. Aryl hydrocarbon receptor-mediated antiestrogenic and antitumorigenic activity of diindolylmethane. *Carcinogenesis*, 19:1631–9.

Chen I, Safe S, Bjeldanes L. 1996. Indole-3-carbinol and diindolylmethane as aryl hydrocarbon (Ah) receptor agonists and antagonists in T47D human breast cancer cells. *Biochem Pharmacol*, 51:1069–76.

Chen Z, Hagler J, Palombella VJ et al. 1995. Signal-induced site-specific phosphorylation targets I kappa B alpha to the ubiquitin-proteasome pathway. *Genes Dev*, 9:1586–97.

Chen ZJ, Parent L, Maniatis T. 1996. Site-specific phosphorylation of IkappaBalpha by a novel ubiquitination-dependent protein kinase activity. *Cell*, 84:853–62.

Chinni SR, Li Y, Upadhyay S et al. 2001. Indole-3-carbinol (I3C) induced cell growth inhibition, G1 cell cycle arrest and apoptosis in prostate cancer cells. *Oncogene*, 20:2927–36.

Chinni SR, Sarkar FH. 2002. Akt inactivation is a key event in indole-3-carbinol-induced apoptosis in PC-3 cells. *Clin Cancer Res*, 8: 1228–36.

Christensen JG, LeBlanc GA. 1996. Reversal of multidrug resistance in vivo by dietary administration of the phytochemical indole-3-carbinol. *Cancer Res*, 56:574–81.

Cory S, Adams JM. 2002. The Bcl2 family: regulators of the cellular life-or-death switch. *Nat Rev Cancer*, 2:647–56.

Cover CM, Hsieh SJ, Tran SH et al. 1998. Indole-3-carbinol inhibits the expression of cyclin-dependent kinase-6 and induces a G1 cell cycle arrest of human breast cancer cells independent of estrogen receptor signaling. *J Biol Chem*, 273:3838–47.

Cram EJ, Liu BD, Bjeldanes LF. 2001. Indole-3-carbinol inhibits CDK6 expression in human MCF-7 breast cancer cells by disrupting Sp1 transcription factor interactions with a composite element in the CDK6 gene promoter. *J Biol Chem*, 276:22332–40.

Cross MJ, Claesson-Welsh L. 2001. FGF and VEGF function in angiogenesis: signalling pathways, biological responses and therapeutic inhibition. *Trends Pharmacol Sci*, 22:201–7.

Dashwood RH, Fong AT, Arbogast DN et al. 1994. Anticarcinogenic activity of indole-3-carbinol acid products: ultrasensitive bioassay by trout embryo microinjection. *Cancer Res*, 54:3617–19.

De Giorgi F, Lartigue L, Bauer MK et al. 2002. The permeability transition pore signals apoptosis by directing Bax translocation and multimerization. *FASEB J*, 16:607–9.

Findley HW, Gu L, Yeager AM et al. 1997. Expression and regulation of Bcl-2, Bcl-xl, and Bax correlate with p53 status and sensitivity to apoptosis in childhood acute lymphoblastic leukemia. *Blood*, 89: 2986–93.

Franke TF, Yang SI, Chan TO et al. 1995. The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell*, 81:727–36.

Frydoonfar HR, McGrath DR, Spigelman AD. 2003. The effect of indole-3-carbinol and sulforaphane on a prostate cancer cell line. *ANZ J Surg*, 73:154–6.

Gao CF, Ren S, Zhang L et al. 2001. Caspase-dependent cytosolic release of cytochrome c and membrane translocation of Bax in p53-induced apoptosis. *Exp Cell Res*, 265:145–51.

Ge X, Fares FA, Yannai S. 1999. Induction of apoptosis in MCF-7 cells by indol-3-carbinol is independent of p53 and bax. *Anticancer Res*, 19:3199–203.

Ge X, Yannai S, Rennert G et al. 1996. 3,3′-Diindolylmethane induces apoptosis in human cancer cells. *Biochem Biophys Res Commun*, 228:153–8.

Ghosh G, van Duyne G, Ghosh S et al. 1995. Structure of NF-kappa B p50 homodimer bound to a kappa B site. *Nature*, 373:303–10.

Haefner B. 2002. NF-kappaB: arresting a major culprit in cancer. *Drug Discov Today*, 7:653–63.

He YH, Smale MH, Schut HA. 1997. Chemopreventive properties of indole-3-carbinol (I3C): inhibition of DNA adduct formation of the dietary carcinogen, 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP), in female F344 rats. *J Cell Biochem Suppl*, 27: 42–51.

Hideshima T, Chauhan D, Richardson P et al. 2002. NF-kappa B as a therapeutic target in multiple myeloma. *J Biol Chem*, 277:16639–47.

Hong C, Firestone GL, Bjeldanes LF. 2002. Bcl-2 family-mediated apoptotic effects of 3,3′-diindolylmethane (DIM) in human breast cancer cells. *Biochem Pharmacol*, 63:1085–97.

Hong C, Kim HA, Firestone GL et al. 2002. 3,3′-Diindolylmethane (DIM) induces a G(1) cell cycle arrest in human breast cancer cells that is accompanied by Sp1-mediated activation of p21(WAF1/CIP1) expression. *Carcinogenesis*, 23:1297–305.

Horn TL, Reichert MA, Bliss RL et al. 2002. Modulations of P450 mRNA in liver and mammary gland and P450 activities and metabolism of estrogen in liver by treatment of rats with indole-3-carbinol. *Biochem Pharmacol*, 64:393–404.

Israel A. 2003. Signal transduction: a regulator branches out. *Nature*, 423:596–7.

Jean D, Bar-Eli M. 2001. Targeting the ATF-1/CREB transcription factors by single chain Fv fragment in human melanoma: potential modality for cancer therapy. *Crit Rev Immunol*, 21:275–86.

Jia L, Patwari Y, Srinivasula SM et al. 2001. Bax translocation is crucial for the sensitivity of leukaemic cells to etoposide-induced apoptosis. *Oncogene*, 20:4817–26.

Jiang H, Su ZZ, Lin JJ et al. 1996. The melanoma differentiation associated gene mda-7 suppresses cancer cell growth. *Proc Natl Acad Sci USA*, 93:9160–5.

Jin L, Qi M, Chen DZ et al. 1999. Indole-3-carbinol prevents cervical cancer in human papilloma virus type 16 (HPV16) transgenic mice. *Cancer Res*, 59:3991–7.

Johnson IT. 2002. Glucosinolates: bioavailability and importance to health. *Int J Vitam Nutr Res*, 72:26–31.

Kane DJ, Sarafian TA, Anton R et al. 1993. Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species. *Science*, 262: 1274–7.

Katchamart S, Williams DE. 2001. Indole-3-carbinol modulation of hepatic monooxygenases CYP1A1, CYP1A2 and FMO1 in guinea pig, mouse and rabbit. *Comp Biochem Physiol C Toxicol Pharmacol*, 129:377–84.

Katdare M, Osborne MP, Telang NT. 1998. Inhibition of aberrant proliferation and induction of apoptosis in pre-neoplastic human mammary epithelial cells by natural phytochemicals. *Oncol Rep*, 5: 311–15.

Kim DJ, Shin DH, Ahn B et al. 2003. Chemoprevention of colon cancer by Korean food plant components. *Mutat Res*, 523–4:99–107.

Kojima T, Tanaka T, Mori H. 1994. Chemoprevention of spontaneous endometrial cancer in female Donryu rats by dietary indole-3-carbinol. *Cancer Res*, 54:1446–9.

Kundu M, Liu PP. 2001. Function of the inv(16) fusion gene CBFB-MYH11. *Curr Opin Hematol*, 8:201–5.

Lake BG, Tredger JM, Renwick AB et al. 1998. 3,3′-Diindolylmethane induces CYP1A2 in cultured precision-cut human liver slices. *Xenobiotica*, 28:803–11.

Lazebnik YA, Kaufmann SH, Desnoyers S et al. 1994. Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature*, 371:346–7.

Le HT, Schaldach CM, Firestone GL et al. 2003. Plant derived 3,3′-diindolylmethane is a strong androgen antagonist in human prostate cancer cells. *J Biol Chem*, 278:21136–45.

Li Y, Li X, Sarkar FH. 2003. Gene expression profiles of I3C- and DIM-treated PC3 human prostate cancer cells determined by cDNA microarray analysis. *J Nutr*, 133:1011–19.

Manson MM, Hudson EA, Ball HW et al. 1998. Chemoprevention of aflatoxin B1-induced carcinogenesis by indole-3-carbinol in rat liver–predicting the outcome using early biomarkers. *Carcinogenesis*, 19:1829–36.

Mendelsohn J. 2002. Targeting the epidermal growth factor receptor for cancer therapy. *J Clin Oncol*, 20:1S–13S.

Meng Q, Goldberg ID, Rosen EM et al. 2000. Inhibitory effects of Indole-3-carbinol on invasion and migration in human breast cancer cells. *Breast Cancer Res Treat*, 63:147–52.

Meng Q, Qi M, Chen DZ et al. 2000. Suppression of breast cancer invasion and migration by indole-3-carbinol: associated with up-regulation of BRCA1 and E-cadherin/catenin complexes. *J Mol Med*, 78:155–65.

Meyskens FL Jr. 1992. Strategies for prevention of cancer in humans. *Oncology (Huntingt)*, 6(2 Suppl):15–24.

Muller CW, Rey FA, Sodeoka M et al. 1995. Structure of the NF-kappa B p50 homodimer bound to DNA. *Nature*, 373:311–17.

Murillo G, Mehta RG. 2001. Cruciferous vegetables and cancer prevention. *Nutr Cancer*, 41:17–28.

Nachshon-Kedmi M, Yannai S, Haj A et al. 2003. Indole-3-carbinol and 3,3'-diindolylmethane induce apoptosis in human prostate cancer cells. *Food Chem Toxicol*, 41:745–52.

Nakayama KI, Hatakeyama S, Nakayama K. 2001. Regulation of the cell cycle at the G1-S transition by proteolysis of cyclin E and p27Kip1. *Biochem Biophys Res Commun*, 282:853–60.

Nho CW, Jeffery E. 2001. The synergistic upregulation of phase II detoxification enzymes by glucosinolate breakdown products in cruciferous vegetables. *Toxicol Appl Pharmacol*, 174:146–52.

Oganesian A, Hendricks JD, Williams DE. 1997. Long term dietary indole-3-carbinol inhibits diethylnitrosamine-initiated hepatocarcinogenesis in the infant mouse model. *Cancer Lett*, 118:87–94.

Orlowski RZ, Baldwin AS. 2002. NF-kappaB as a therapeutic target in cancer. *Trends Mol Med*, 8:385–9.

Ozes ON, Mayo LD, Gustin JA et al. 1999. NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase. *Nature*, 401:82–5.

Park JR, Hockenbery DM. 1996. BCL-2, a novel regulator of apoptosis. *J Cell Biochem*, 60:12–17.

Philpott NJ, Turner AJ, Scopes J et al. 1996. The use of 7-amino actinomycin D in identifying apoptosis: simplicity of use and broad spectrum of application compared with other techniques. *Blood*, 87: 2244–51.

Platten M, Wick W, Weller M. 2001. Malignant glioma biology: role for TGF-beta in growth, motility, angiogenesis, and immune escape. *Microsc Res Tech*, 52:401–10.

Putcha GV, Deshmukh M, Johnson EM Jr. 1999. BAX translocation is a critical event in neuronal apoptosis: regulation by neuroprotectants, BCL-2, and caspases. *J Neurosci*, 19:7476–85.

Rahman KM, Aranha O, Glazyrin A et al. 2000. Translocation of Bax to mitochondria induces apoptotic cell death in indole-3-carbinol (I3C) treated breast cancer cells. *Oncogene*, 19:5764–71.

Rahman KW, Aranha O, Sarkar FH. 2003. Indole-3-Carbinol (I3C) induces apoptosis in tumorigenic but not in nontumorigenic breast epithelial cells. *Nutr Cancer*, 45:101–12.

Riby JE, Feng C, Chang YC et al. 2000. The major cyclic trimeric product of indole-3-carbinol is a strong agonist of the estrogen receptor signaling pathway. *Biochemistry*, 39:910–18.

Romashkova JA, Makarov SS. 1999. NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling. *Nature*, 401:86–90.

Romier C, Cocchiarella F, Mantovani R et al. 2003. The NF-YB/NF-YC structure gives insight into DNA binding and transcription regulation by CCAAT factor NF-Y. *J Biol Chem*, 278:1336–45.

Rommel C, Clarke BA, Zimmermann S et al. 1999. Differentiation stage-specific inhibition of the Raf-MEK-ERK pathway by Akt. *Science*, 286:1738–41.

Salomons GS, Brady HJ, Verwijs-Janssen M et al. 1997. The Bax alpha: Bcl-2 ratio modulates the response to dexamethasone in leukaemic cells and is highly variable in childhood acute leukaemia. *Int J Cancer*, 71:959–65.

Sebolt-Leopold JS. 2000. Development of anticancer drugs targeting the MAP kinase pathway. *Oncogene*, 19:6594–9.

Seger R, Krebs EG. 1995. The MAPK signaling cascade. *FASEB J*, 9: 726–35.

Steinmetz KA, Potter JD. 1996. Vegetables, fruit, and cancer prevention: a review. *J Am Diet Assoc*, 96:1027–39.

Stresser DM, Bailey GS, Williams DE. 1994. Indole-3-carbinol and beta-naphthoflavone induction of aflatoxin B1 metabolism and cytochromes P-450 associated with bioactivation and detoxication of aflatoxin B1 in the rat. *Drug Metab Dispos*, 22:383–91.

Takahashi N, Dashwood RH, Bjeldanes LF et al. 1995. Mechanisms of indole-3-carbinol (I3C) anticarcinogenesis: inhibition of aflatoxin B1-DNA adduction and mutagenesis by I3C acid condensation products. *Food Chem Toxicol*, 33:851–7.

Tawfiq N, Heaney RK, Plumb JA et al. 1995. Dietary glucosinolates as blocking agents against carcinogenesis: glucosinolate breakdown products assessed by induction of quinone reductase activity in murine hepa1c1c7 cells. *Carcinogenesis*, 16:1191–4.

Traenckner EB, Pahl HL, Henkel T et al. 1995. Phosphorylation of human I kappa B-alpha on serines 32 and 36 controls I kappa B-alpha proteolysis and NF-kappa B activation in response to diverse stimuli. *EMBO J*, 14:2876–83.

van Iersel ML, Verhagen H, van Bladeren PJ. 1999. The role of biotransformation in dietary (anti)carcinogenesis. *Mutat Res*, 443: 259–70.

Vang O, Jensen MB, Autrup H. 1990. Induction of cytochrome P450IA1 in rat colon and liver by indole-3-carbinol and 5,6-benzoflavone. *Carcinogenesis*, 11:1259–63.

Verhoeven DT, Verhagen H, Goldbohm RA et al. 1997. A review of mechanisms underlying anticarcinogenicity by brassica vegetables. *Chem Biol Interact*, 103:79–129.

Verkerk R, van der Gaag MS, Dekker M et al. 1997. Effects of processing conditions on glucosinolates in cruciferous vegetables. *Cancer Lett*, 114:193–4.

Verma IM, Stevenson JK, Schwarz EM et al. 1995. Rel/NF-kappa B/I kappa B family: intimate tales of association and dissociation. *Genes Dev*, 9:2723–35.

Wong GY, Bradlow L, Sepkovic D et al. 1997. Dose-ranging study of indole-3-carbinol for breast cancer prevention. *J Cell Biochem Suppl*, 28–29:111–16.

Wortelboer HM, De Kruif CA, van Iersel AA et al. 1992. Acid reaction products of indole-3-carbinol and their effects on cytochrome P450 and phase II enzymes in rat and monkey hepatocytes. *Biochem Pharmacol*, 43:1439–47.

Yamamoto Y, Verma UN, Prajapati S et al. 2003. Histone H3 phosphorylation by IKK-alpha is critical for cytokine-induced gene expression. *Nature*, 423:655–9.

Yang CS, Landau JM, Huang MT et al. 2001. Inhibition of carcinogenesis by dietary polyphenolic compounds. *Annu Rev Nutr*, 21:381–406.

Yasui K, Arii S, Zhao C et al. 2002. TFDP1, CUL4A, and CDC16 identified as targets for amplification at 13q34 in hepatocellular carcinomas. *Hepatology*, 35:1476–84.

Zheng Q, Hirose Y, Yoshimi N et al. 2002. Further investigation of the modifying effect of various chemopreventive agents on apoptosis and cell proliferation in human colon cancer cells. *J Cancer Res Clin Oncol*, 128:539–46.

SELECTIVE GROWTH REGULATORY AND PRO-APOPTOTIC EFFECTS OF DIM IS MEDIATED BY AKT AND NF-kappaB PATHWAYS IN PROSTATE CANCER CELLS

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1. ABSTRACT

Prostate cancer is the second leading cause of cancer related deaths in men in the United States. I3C and its *in vivo* dimeric product, DIM, have been found to inhibit the growth of prostate cancer cells. However, the molecular mechanism(s) by which DIM elicits its effects on prostate cancer cells has not been fully elucidated. We have previously shown that I3C induces apoptosis and inhibits the activation of NF-kappaB pathway, which could be mediated via Akt signaling pathway. In this study, we investigated whether there is any cross-talk between Akt and NF-kappaB during DIM-induced apoptosis in PC-3 prostate cancer cells. We found that DIM inhibited cell growth and induced apoptosis in PC-3 prostate cancer cells but not in non-tumorigenic CRL2221 human prostate epithelial cells. DIM also inhibited EGFR expression, PI3K kinase activity, and Akt activation, and abrogated the EGF-induced activation of PI3K in prostate cancer cells. NF-kappaB DNA-binding analysis and transfection studies with Akt cDNA constructs revealed that Akt transfection resulted in the induction of NF-kappaB activity and this was inhibited by DIM treatment. DIM treatment also showed significant induction of apoptosis in non-transfected cells compared to Akt and Akt-Myr transfected prostate cancer cells. From these results, we conclude that the inhibition of Akt and NF-kappaB activity and their cross-talk is a novel mechanism by which DIM inhibits cell growth and induces apoptotic processes in prostate cancer cells but not in non-tumorigenic prostate epithelial cells.

2. INTRODUCTION

Prostate cancer is one of the most common cancers in men and the second leading cause of male cancer death in the United States (1). However, Asians have relatively low incidence of prostate cancers. Dietary and epidemiological studies have shown an association between high dietary intake of vegetables and decreased prostate cancer risk (2, 3). Among vegetables with anticarcinogenic properties, the cruciferous vegetable family including broccoli, cabbage, brussels sprouts, and cauliflower appears to be most effective at reducing the risk of cancers. Indole-3-carbinol (I3C), a common phytochemical in the human diet, is present in almost all members of the cruciferous vegetable family, and it is readily converted to its dimeric product, 3,3'-diinolylmethane (DIM) (4). There are growing evidences showing that I3C and DIM have the potential to inhibit a number of common cancers, especially those that are hormone-related (5-8).

It has been demonstrated that I3C and DIM possess anti-carcinogenic effects in experimental animals and inhibits the growth of human cancer cells *in vitro* (5, 7-10). DIM has been found to induce cell cycle arrest at G1 phase with up-regulation of $p21^{WAF1}$ and down-regulation of CDK6 (6, 11). It has been reported that DIM increases the expression of Bax, decreases the expression of Bcl-2, and induces apoptosis (12). Because of these effects, the interest in I3C and DIM as cancer chemopreventive and/or

therapeutic agents has significantly increased in the past years. We have previously shown that I3C up-regulates $p21^{WAF1}$, Bax, and $p27^{KIP1}$, and down-regulates Bcl_{XL}, EGFR, and Akt kinase activity, leading to the induction of apoptosis in prostate cancer cells (13, 14). We have also reported the gene expression profiles of prostate cancer cells exposed to I3C and DIM, showing that I3C and DIM induce the expression of genes related to the Phase I and Phase II enzymes and regulate the expression of genes involved in the control of cell growth, cell cycle, apoptosis, signal transduction, and oncogenesis (15). However, the precise molecular mechanism by which DIM exerts its effect on the induction of apoptosis and the cell signaling pathways, has not been fully elucidated.

Akt and NF-kappaB pathways are important cell signaling pathways involved in the processes of apoptosis, carcinogenesis, and tumor progression (16-19). NF-kappaB is a cell survival factor and can be activated by many types of stimuli including TNF-α, EGF, UV radiation, etc. There is growing evidence to suggest the role of NF-kappaB in the protection against apoptosis (18, 20). An *in vivo* study showed that mice lacking NF-kappaB p65/RelA died embryonically from extensive apoptosis in the liver (21), suggesting anti-apoptotic role of NF-kappaB. Akt can be activated by various growth factors including EGF through activation of phosphatidylinositol kinase (PI3K) (17). Activated Akt functions to promote cell survival by inhibiting apoptosis through its ability to phosphorylate and inactivate downstream targets (16, 17). Several reports have showed that Akt also regulates the NF-kappaB pathway via phosphorylation and activation of molecules in the NF-kappaB pathway (22-24). Because both Akt and NF-kappaB have been critically involved in the cell survival and apoptotic process in this study we investigated whether DIM could inhibit Akt and NF-kappaB activation leading to apoptosis, and whether Akt and NF-kappaB pathways could cross-talk during apoptotic process induced by DIM. Finally, we also investigated whether there is any differential effect of DIM between PC-3 prostate cancer cells and non-tumorigenic CRL2221 prostate epithelial cells.

3. MATERIALS AND METHODS

3.1. Cell culture and reagents

PC-3 human prostate cancer cells (ATCC, Manassas, VA) were cultured in RPMI-1640 media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin in a 5% CO_2 atmosphere at 37°C. CRL-2221 human non-tumorigenic prostate epithelial cells (ATCC, Manassas, VA) were cultured in keratinocyte-SFM media (Invitrogen, Carlsbad, CA) supplemented with EGF (0.2 microgram/L), bovine pituitary extract (30 milligram/L), and 1% penicillin and streptomycin. DIM (LKT, St. Paul, Minnesota) was dissolved in DMSO to make 60 millimole/L stock solution. Wherever indicated, EGF (Invitrogen, Carlsbad, CA) was added to the media at a final concentration of 100 microgram/L.

3.2. Cell growth inhibition by MTT assay

The PC-3 and CRL-2221 cells were seeded at a density of 1×10^3/well in 96 well culture dishes. After 24 hours, the cells were treated with 15, 30, and 60 micromole/L DIM for one to three days. Control PC-3 cells received 0.1% DMSO for same time points. The cells were then incubated with MTT (0.5 gram/L, Sigma, St. Louis, MO) at 37°C for 4h and with DMSO at room temperature for 1 h. The spectrophotometric absorbance of the samples was measured by using ULTRA Multifunctional Microplate Reader (TECAN, Durham, NC) at 595 nm. The experiment was repeated three times and t test was performed to verify the significance of cell growth inhibition after treatment.

3.3. Histone/DNA ELISA for detecting apoptosis

Cell Apoptosis ELISA Detection Kit (Roche, Palo Alto, CA, USA) was used to detect apoptosis in PC-3 and CRL-2221 cells with different treatments according to manufacturer's protocol. Briefly, the cytoplasmic histone/DNA fragments from PC-3 and CRL-2221 cells treated with 15, 30, and 60 micromole/L DIM or 0.1% DMSO (vehicle control) for 24, 48, 72 hours, were extracted and bound to immobilized anti-histone antibody. Subsequently, the peroxidase-conjugated anti-DNA antibody was used for the detection of immobilized histone/DNA fragments. After addition of substrate for peroxidase, the spectrophotometric absorbance of the samples was determined by using ULTRA Multifunctional Microplate Reader (TECAN, Durham, NC) at 405 nm.

3.4. Western blot analysis

The PC-3 and CRL-2221 cells were plated on culture dishes and allowed to attach for 24 hours followed by the treatment with 15, 30, or 60 micromole/L DIM for 48 hours. Control cells were incubated in the medium with 0.1% DMSO using same time points. After incubation, the cells were lysed in 62.5 millimole/L Tris-HCl and 2% SDS. Protein concentration was then measured using BCA protein assay (PIERCE, Rockford, IL). Cell extracts were subjected to 10% SDS-PAGE, and electrophoretically transferred to nitrocellulose membrane. Membranes were incubated with anti-Akt (Santa Cruz Biotech, Santa Cruz, CA), anti-phospho-Akt Ser473 (Cell signaling, Beverly, MA), anti-EGFR (Santa Cruz Biotech, Santa Cruz, CA), anti-Bcl_{XL} (Santa Cruz Biotech, Santa Cruz, CA), and anti-β-actin (Sigma, St. Louis, MO) antibodies, washed with TTBS and incubated with secondary antibody conjugated with peroxidase. The signal was then detected using the chemiluminescent detection system (PIERCE, Rockford, IL).

3.5. Reporter gene constructs and transfection

pLNCX-Akt (normal Akt), pLNCX-Myr-Akt (constitutively activated Akt), pLNCX-Akt-K179M (dominant negative), and pLNCX (control vector) were generously provided by Dr. Sellers (Dana-Farber Cancer Institute, Boston, MA). NF-kappaB-LUC (Stratagene, La Jolla, CA) contains six repeated copies of the NF-kappaB DNA-binding site and a luciferase reporter gene. CMV-beta-gal reporter construct transfection was used for normalization of transfection efficiency. The pLNCX-Akt,

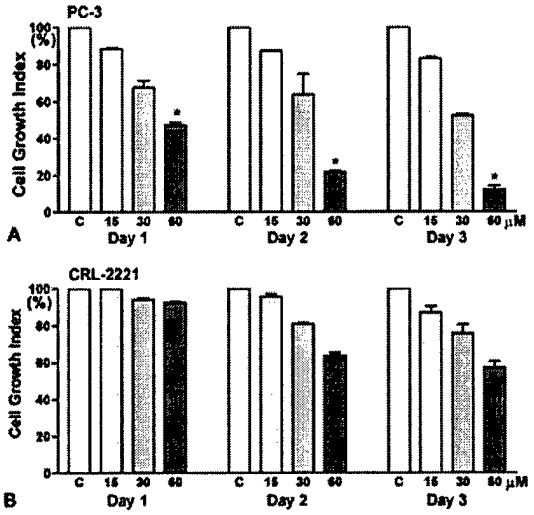

Figure 1. Effects of DIM on the growth of PC-3 (A) and CRL-2221 cells (B) tested by MTT assay. (*: $p<0.05$; n=3).

pLNCX-Myr-Akt, pLNCX-Akt-K179M, or pLNCX was transiently co-transfected with NF-kappaB-LUC and CMV-beta-gal into PC-3 cells when they were at ~70% confluent using the LipofectAMINE (Invitrogen, Carlsbad, CA). After incubation for 5 hours, the transfected cells were washed and incubated with RPMI-1640 media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum overnight followed by treatment with 60 micromole/L DIM for 48 hours. Subsequently, the luciferase activities in the samples were measured by using Steady-Glo™ Luciferase assay system (Promega, Madison, WI) and ULTRA Multifunctional Microplate Reader (TECAN, Durham, NC). To detect the NF-kappaB activity in stably transfected PC-3 cells, the samples were subjected to NF-kappaB DNA-binding activity measurement using EMSA method as described below. Cell Apoptosis ELISA Detection Kit was also used to detect apoptosis in transfected and parental PC-3 cells.

3.6. NF-kappaB DNA-binding activity measurement

PC-3 and CRL-2221 cells were treated with 15, 30, and 60 micromole/L DIM for 72 hours. Following treatment, the nuclear proteins from cells were extracted. Ten microgram of nuclear proteins was subjected to electrophoretic mobility shift assay (EMSA) as described previously (25). Competition assay using unlabeled specific competitor (NF-kappaB oligo) was conducted to confirm the specificity of NF-kappaB DNA-binding activity.

3.7. Immunoprecipitation and PI3K kinase assay

The PI3K kinase activity of PC-3 cells treated with 60 micromole/L DIM for 24 and 48 hours, 60 micromole/L DIM for 24 and 48 hours followed by EGF treatment for 20 minutes, EGF only, or 0.1% DMSO was measured by using PI3K kinase assay kit (Echelon, Salt Lake, UT) according to manufacturer's protocol. Briefly, the cells after treatments were lysed in ice-cold cell lysis buffer (137 millimole/L NaCl, 20 millimole/L Tris-HCl, 1 millimole/L $CaCl_2$, 1 millimole/L $MgCl_2$, 0.1 millimole/L sodium orthovanadate, 1% NP-40, and 1 millimole/L PMSF) on ice for 20 minutes. After centrifugation, the protein concentration of supernatant was measured by BCA protein assay (PIERCE, Rockford, IL). 600 micrograms of proteins from each sample were used for immunoprecipitation with PI3K antibody (Upstate, Charlottesville, VA) overnight and protein G-agarose for one hour at 4°C. Then, the samples were collected by centrifugation, washed with kinase buffer, and subjected to PI3K kinase assay in kinase buffer (20 millimole/L Tris pH 7.4, 4 millimole/L $MgCl_2$, 10 millimole/L NaCl), 25 micromole/L ATP, and 2.4 micrograms of $PI(4,5)P_2$ as kinase substrate. PI(3,4,5)P3 was measured by competitive ELISA.

3.8. Signal quantification and statistical analysis

The EMSA gel was scanned, and the signals in the gel were quantified and analyzed with Odyssey software (LI-COR, Lincoln, NE). Signal in the Western blots was also scanned and quantified with Molecular Analyst software (Bio-Rad, Hercules, CA). Comparisons were made between control and treatments. Statistical analysis was performed using t test between treated and untreated samples. P values less than 0.05 were used to indicate statistical significance.

4. RESULTS

4.1. DIM selectively inhibits growth of prostate cancer cells

PC-3 prostate cancer cells and CRL-2221 non-tumorigenic human prostate epithelial cells were treated with 0-60 micromole/L DIM over 3 days and the cell viability was determined by MTT assay. The treatment of PC-3 prostate cancer cells with DIM resulted in a dose and time-dependent inhibition of cell proliferation (Figure 1A). However, only 37.8% growth inhibition was observed in the CRL-2221 non-tumorigenic prostate epithelial cells treated with 60 micromole/L DIM for 3 days compared to 87.8% in PC-3 cells (Figure 1B), suggesting the selective growth inhibition of prostate cancer cells by DIM. Inhibition of cell proliferation observed by MTT could be partly due to the induction of apoptosis in prostate cancer cells. We, therefore, investigated whether DIM could selectively induce apoptosis in PC-3 prostate cancer cells.

4.2. DIM selectively induces apoptosis in prostate cancer cells

By ELISA analysis of cytoplamic histone/DNA fragments, we observed an induction of apoptosis in prostate cancer cells treated with 15-60 micromole/L DIM (Figure 2A). The induction of apoptosis was time- and dose-dependent, and was directly correlated with the inhibition of cell growth, suggesting that DIM treatment may result in the inhibition of cell proliferation through apoptotic cell death. More importantly, non-tumorigenic CRL-2221 prostate epithelial cells were much less responsive to DIM treatment than PC-3 cells (Figure 2B), suggesting that DIM selectively induced apoptosis in prostate cancer cells.

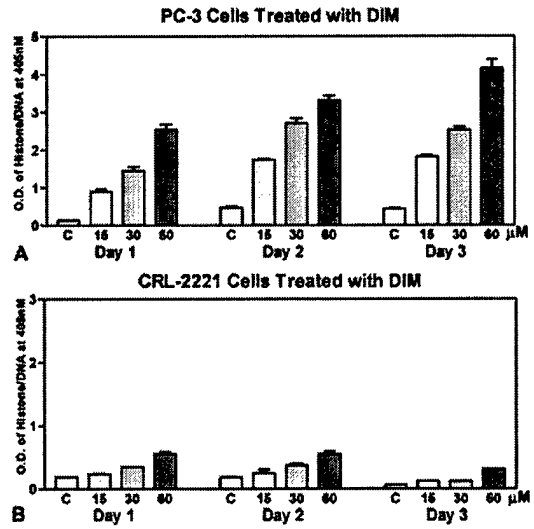

Figure 2. Induction of apoptosis in PC-3 (A) and CRL-2221 (B) cells tested by ELISA. (n=2).

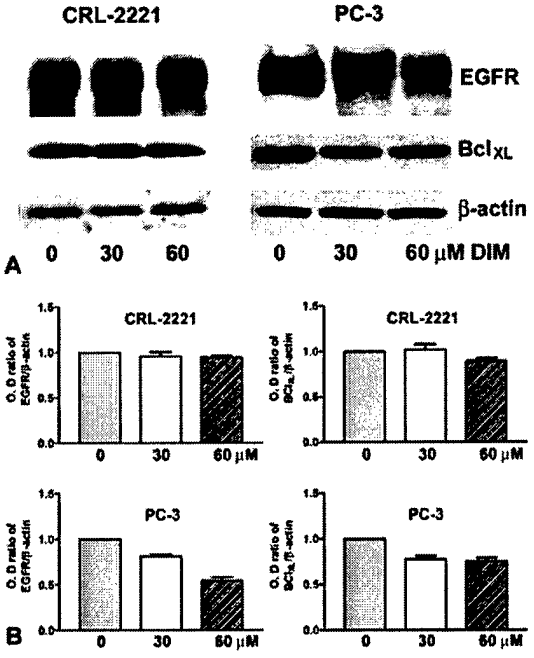

Figure 3. Western blot (A) and densitometric analysis (B) of Bcl_{XL} and EGFR in PC-3 and CRL-2221 cells treated with DIM.

By Western Blot analysis, we also found that DIM inhibited the expression of Bcl_{XL}, an anti-apoptotic protein in PC-3 cells (Figure 3). However, there was no significant effect on Bcl_{XL} in DIM-treated CRL-2221 cells, and these results were correlated with minimal apoptosis in DIM-treated CRL-2221 cells. Next, we investigated whether PI3K/Akt and NF-kappaB signaling pathways are involved in the apoptotic processes induced by DIM in prostate cancer cells.

4.3. DIM inhibits PI3K and Akt activation and induces apoptoisis through Akt pathway

Since Akt signaling pathway is an important signal transduction pathway that plays a critical role in cell survival and apoptotic processes, we investigated the status of Akt in PC-3 and CRL-2221 cells treated with 0-60 micromole/L DIM by Western Blot analysis. We did not find any alterations in the protein expression of un-phosphorylated Akt in DIM-treated PC-3 and CRL-2221 cells (Figure 4). However, a significant decrease in the phosphorylated Akt protein at Ser473 was observed in DIM treated PC-3 cells compared to control cells, suggesting inactivation of Akt kinase after DIM treatment (Figure 4). Treatment of DIM showed dose dependent inhibition of Akt phosphorylation in PC-3 cells, consistent with the induction of apoptosis by DIM. However, the phosphorylated Akt Ser473 protein was undetectable in CRL-2221 cells treated or untreated with DIM, suggesting that the inactivation of Akt by DIM is specific in prostate cancer cells compared to non-tumorigenic prostate epithelial cells.

Since Akt is activated through the activation of PI3K, we investigated the PI3K kinase activity in the PC-3 cells treated with DIM or pre-treated with DIM followed by EGF stimulation. We found that DIM treatment inhibited the activity of PI3K (Figure 5), suggesting that DIM could inactivate Akt through the inhibition of PI3K activity. We also found that EGF treatment alone activated PI3K kinase activity as expected, and that DIM pretreatment abrogated the activation of PI3K stimulated by EGF (Figure 5). Because the activation of Akt and PI3K could be mediated through EGFR pathways, we measured the expression of EGFR by Western Blot analysis. We found that DIM inhibited EGFR expression in a dose-dependent manner in PC-3 cells only and showed no inhibition of EGFR in CRL-2221 cells (Figure 3), corresponding with the selective effect of DIM on Akt activation in PC-3 cells.

Furthermore, we transfected Akt cDNA into PC-3 cells and measured the degree of apoptosis in transfected cells with and without DIM treatment. We found that DIM not only induced apoptosis in PC-3 parental cells but also in Akt transfected PC-3 cells although to a lesser extent as expected (Figure 6). More importantly, we found that transfection of constitutively activated Akt (pLNCX-Myr-Akt) and wild-type Akt (pLNCX-Akt) inhibited apoptosis induced by DIM compared to mutant Akt and empty vector transfectants, suggesting that the induction of apoptosis by DIM is partly mediated by active Akt and that the overexpression of Akt leads to resistance to DIM-induced apoptosis.

4.4. DIM selectively inhibits NF-kappaB activation in PC-3 cells

Nuclear extracts from control and DIM-treated PC-3 cells were subjected to NF-kappaB DNA-binding activity as measured by EMSA. Autoradiography revealed that 30-60 micromole/L DIM significantly inhibited NF-kappaB DNA-binding activity in PC-3 cells compared to untreated control (Figure 7). However, no such effect was observed in DIM-treated CRL-2221 cells, suggesting the

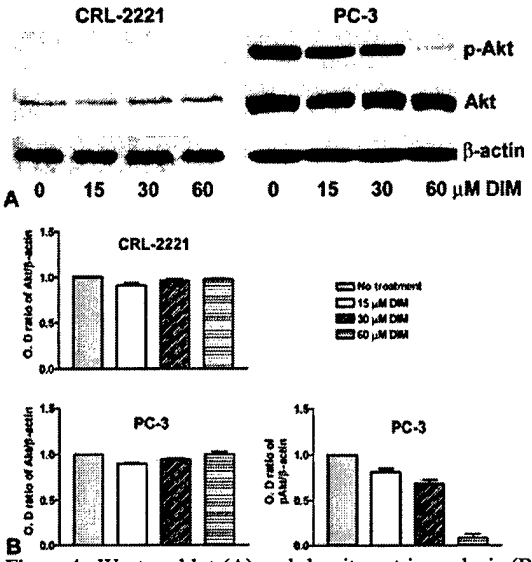

Figure 4. Western blot (A) and densitometric analysis (B) of total Akt and p-Akt in PC-3 and CRL-2221 cells treated with DIM.

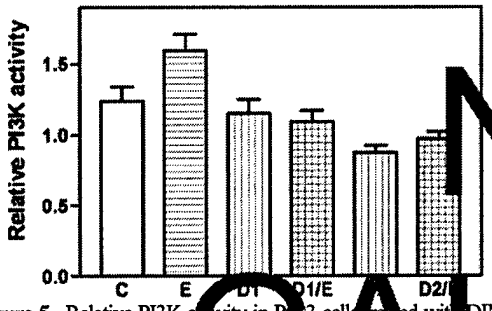

Figure 5. Relative PI3K activity in PC-3 cells treated with DIM. (C: control; E: treated with EGF; D1: treated with 60 micromole/L DIM for 24 hours; D1/E: treated with 60 micromole/L DIM for 24 hours followed by EGF treatment; D2: treated with 60 micromole/L DIM for 48 hours; D2/E: treated with 60 micromole/L DIM for 48 hours followed by EGF treatment).

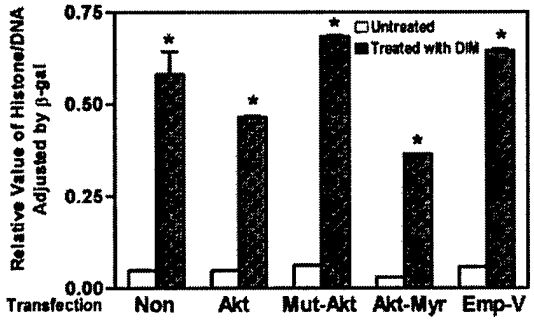

Figure 6. Induction of apoptosis in transfected and DIM treated PC-3 cells tested by ELISA (Non: no transfection; Akt: transfected with pLNCX-Akt; Mut-Akt: transfected with pLNCX-Akt-K179M; Akt-Myr: transfected with pLNCX-Myr-Akt; Emp-V: transfected with empty vector, pLNCX; *: $p<0.05$; n=2).

selective inhibitory effect of DIM on NF-kappaB DNA-binding activity in prostate cancer cells. In order to further explore the inhibitory effects of DIM on Akt and NF-kappaB pathways, we conducted transfection experiments as described under Materials and Methods. Luciferase assay showed a significant increase in luciferase activity in PC-3 cells co-transfected with pLNCX-Myr-Akt and NF-kappaB-Luc, and also in PC-3 cells co-transfected with pLNCX-Akt and NF-kappaB-Luc (Fig 8). Moreover, DIM significantly abrogated the induction of luciferase activity caused by pLNCX-Myr-Akt and pLNCX-Akt transfections (Figure 8).

To confirm these results, we also examined the NF-kappaB DNA-binding activity in PC-3 cells transfected with pLNCX-Myr-Akt or pLNCX. We observed an increase in NF-kappaB DNA-binding activity in PC-3 cells transfected with pLNCX-Myr-Akt (Figure 9). We also found that DIM abrogated the activation of NF-kappaB DNA-binding activity caused by pLNCX-Myr-Akt transfection (Figure 9). Collectively, these results provide evidence for a potential cross-talk between Akt and NF-kappaB pathways during DIM induced cell growth inhibition and apoptosis in prostate cancer cells.

5. DISCUSSION

DIM, the major *in vivo* product of dietary I3C, has been shown to inhibit cell growth and induce apoptosis in breast, cervical and prostate cancer cells (5-7, 11), suggesting its chemopreventive and/or therapeutic effects on cancer cells. However, the precise molecular mechanisms by which DIM inhibits cell growth and induces apoptosis have not been fully elucidated. Additionally, the effect of DIM on non-tumorigenic epithelial cells remains unknown. Here, we demonstrated that DIM significantly and selectively inhibited cell growth and induced apoptosis in PC-3 prostate cancer cells, while CRL-2221 non-tumorigenic cells showed much less apoptosis by DIM. These results provide evidence for selective effects of DIM on cell growth and apoptosis in cancer cells. To discover the molecular mechanisms responsible for the induction of apoptosis by DIM, we investigated the effects of DIM on Akt and NF-kappaB pathways, which have been known to play important roles in cell survival and apoptotic cell death processes.

It has been known that Akt signaling pathway can be activated by various growth and survival factors such as EGF, PDGF, insulin, etc, through activation of PI3K (16, 17). PI3K activation leads to the production of phosphatidylinositol-3,4,5-trisphosphate ($PI\text{-}3,4,5\text{-}P_3$), which interacts with Akt PH domain. This interaction subsequently causes conformational changes in Akt, resulting in the exposure of two main phosphorylation sites in Akt. Akt is then activated by phosphorylation at Thr308 by Phosphoinositide-dependent protein kinase 1 (PDK1) or at Ser473 by PDK2. Activated Akt functions to promote cell survival by inhibiting apoptosis through its ability to phosphorylate and inactivate several targets including Bad, Forkhead transcription factors, and caspase-9, all of which are involved in apoptotic pathway (26, 27). In the apoptotic

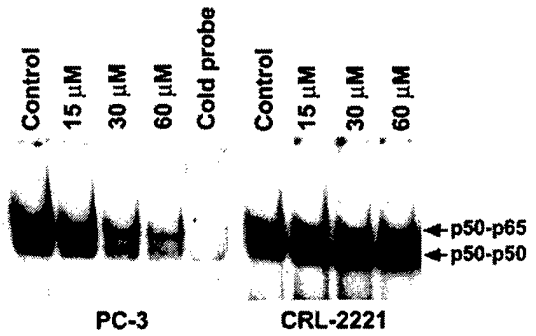

Figure 7. NF-kappaB DNA-binding activity in DIM treated PC-3 and CRL-2221cells tested by EMSA. (Cold probe: Unlabeled NF-kappaB oligonucleotide was used as specific competitor in DNA-binding reaction).

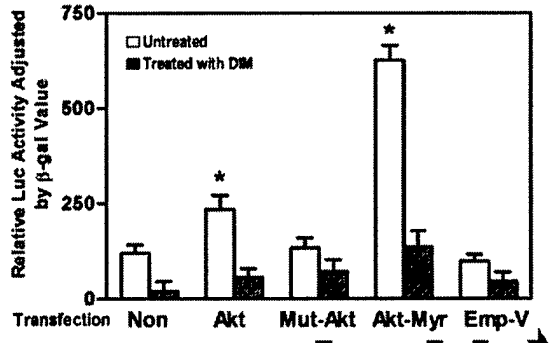

Figure 8. Luciferase activity in transfected PC-3 cells with or without DIM treatments (Non: no transfection; Akt: transfected with pLNCX-Akt; Mut-Akt: transfected with pLNCX-Akt-K179M; Akt-Myr: transfected with pLNCX-Myr-Akt; Emp-V: transfected with empty vector, pLNCX; *: $p<0.05$; n=2).

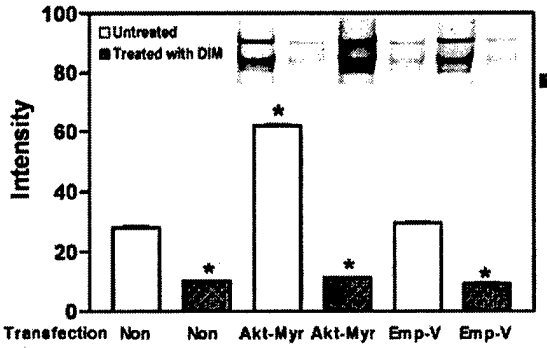

Figure 9. EMSA and densitometric analysis of NF-kappaB DNA-binding activity in transfected PC-3 cells with or without DIM treatments (Non: no transfection; Akt-Myr: transfected with pLNCX-Myr-Akt; Emp-V: transfected with empty vector, pLNCX; *: $p<0.05$; n=2).

process, dephosphorylated Bad (activated form of Bad) translocates to mitochondria, where it heterodimerizes via its BH3 domain with anti-apoptotic BCL family members such as Bcl-2 and Bcl_{XL}, promoting the onset of apoptosis (28-31). In this study, we found that DIM treatment caused the down-regulation of EGFR, suggesting that DIM could inhibit the activation of PI3K through decrease in growth factor binding. By PI3K kinase assay, we found that DIM inactivated PI3K and abrogated the activation of PI3K caused by EGF, suggesting that DIM could inhibit Akt activity by inactivation of PI3K. Subsequently, we found no changes in the expression of un-phosphorylated Akt in DIM treated PC-3 cells. However, DIM decreased the level of phosphorylated Akt, which is the activated form of Akt. This could subsequently increase the activated Bad binding to Bcl-2 and Bcl_{XL}. We found that DIM inhibited the expression of Bcl_{XL}, suggesting an increase in the ratio of Bad/Bcl_{XL}, which could promote cancer cell to apoptotic cell death. Indeed, we observed significant induction of apoptosis in DIM treated PC-3 prostate cancer cells, and lesser apoptosis in Akt transfected PC-3 cells compared to parental PC-3 cells. These results suggest that the inhibition of PI3K/Akt signaling pathway by DIM is one of the mechanisms by which DIM induces apoptosis in prostate cancer cells.

It has been well known that NF-kappaB plays an important role in the apoptotic process (18, 20). Thus, DIM may induce apoptosis by modulating multiple components in the Akt and NF-kappaB pathways. In this study, we found that DIM selectively inhibited NF-kappaB DNA-binding activity in PC-3 prostate cancer cells. The inactivation of NF-kappaB DNA-binding activity may be another mechanism by which DIM induces apoptosis in PC-3 cells. It has been reported that the activity of NF-kappaB may be regulated by a variety of factors including Akt (22, 24). Akt has been shown to enhance the degradation of the IkappaB and induce NF-kappaB activation (32). The ability of Akt to regulate NF-kappaB activity may be through direct interaction with the IKK, supported by the observation that Akt is associated with the IKK complex *in vivo* (33). It has been demonstrated that Akt can phosphorylate and activate IKK at a critical regulatory site, Thr23, and subsequently activate NF-kappaB (24). In this study, we transfected Akt to PC-3 prostate cancer cells and tested the effect of Akt on NF-kappaB DNA-binding activity by EMSA and luciferase assay. Our results showed that Akt regulated NF-kappaB activation, and this observation is in accordance with our published data (25). Importantly, we found that DIM abrogated the NF-kappaB activation stimulated by Akt transfection, suggesting that the inhibition of NF-kappaB activity by DIM is partly mediated through Akt signaling pathway. Hence, the inactivation of DNA-binding activity of NF-kappaB by DIM appears to be responsible for DIM-induced apoptosis in PC-3 prostate cancer cells.

NF-kappaB has been described as a major culprit in cancer (34), and Akt has been known as a key molecule in cell survival (16, 17). Because of their importance in the control of cell survival and apoptotic cell death, both Akt and NF-kappaB have been believed to be very attractive therapeutic targets for cancer therapy (33, 35-37). Therefore, our results indicate that the inhibition of Akt and NF-kappaB activity could be easily achievable by DIM treatment, which inhibits cell growth and induces apoptosis in PC-3 prostate cancer cells, suggesting that DIM may be a useful agent for the prevention and/or treatment of

prostate cancer. More importantly, we found that DIM had no significant effects on cell growth, apoptosis, Akt and NF-kappaB activity in non-tumorigenic prostate epithelial cells, suggesting cancer cell specific effects of DIM. Similar cancer cell specific effects of I3C were also reported previously by our laboratory in breast epithelial cells (38). The fact that DIM selectively inhibits cell growth, Akt and NF-kappaB activation, and induces apoptosis in PC-3 prostate cancer cells, makes it a potent chemopreventive and/or therapeutic agent against prostate cancer. Our results warrant further animal and human investigations in order to fully appreciate the value of DIM in human health.

6. ACKNOWLEDGEMENT

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7. REFERENCES

1. Jemal A., T.Murray, A.Samuels, A.Ghafoor, E.Ward & M.J.Thun: Cancer statistics, 2003. *CA Cancer J Clin* 53,5-26 (2003)

2. Cohen J.H., A.R.Kristal & [illegible] and vegetable intakes and prostate cancer risk. *J [illegible] Inst* 92,61-68 (2000)

3. Verhoeven D.T., R.A.Gol[illegible]ohm, G.v[illegible]ppel, H.Verhagen & P.A.van den [illegible]ndt: Epidemiological studies on brassica vegetables and cancer risk. *Cancer Epidemiol Biomarkers Prev* 5,733-748 (1996)

4. Broadbent T.A. & H.S.Broadbent: 1. The chemistry and pharmacology of indole-3-[illegible] (indole-[illegible]methane) and 3-(methoxymethyl)indol[illegible] [Part II]. *Curr [illegible]Chem* 469-491 (1998)

5. Chen D.Z., M.Qi, K.[illegible] Auborn [illegible] T.A.Carter: Indole-3-carbinol and diindolylmeth[illegible]duce apoptosis of [illegible] cervical cancer cells and in murine HPV16-transgenic preneoplastic cervical epithelium. *J Nutr* 131,3294-3302 (2001)

6. Firestone G.L. & L.F.Bjeldanes: Indole-3-carbinol and 3-3'-diindolylmethane antiproliferative signaling pathways control cell-cycle gene transcription in human breast cancer cells by regulating promoter-Sp1 transcription factor interactions. *J Nutr* 133,2448S-2455S (2003)

7. Nachshon-Kedmi M., S.Yannai, A.Haj & F.A.Fares: Indole-3-carbinol and 3,3'-diindolylmethane induce apoptosis in human prostate cancer cells. *Food Chem Toxicol* 41,745-752 (2003)

8. He Y.H., M.D.Friesen, R.J.Ruch & H.A.Schut: Indole-3-carbinol as a chemopreventive agent in 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) carcinogenesis: inhibition of PhIP-DNA adduct formation, acceleration of PhIP metabolism, and induction of cytochrome P450 in female F344 rats. *Food Chem Toxicol* 38,15-23 (2000)

9. Ge X., S.Yannai, G.Rennert, N.Gruener & F.A.Fares: 3,3'-Diindolylmethane induces apoptosis in human cancer cells. *Biochem Biophys Res Commun* 228,153-158 (1996)

10. Chen I., S.Safe & L.Bjeldanes: Indole-3-carbinol and diindolylmethane as aryl hydrocarbon (Ah) receptor agonists and antagonists in T47D human breast cancer cells. *Biochem Pharmacol* 51,1069-1076 (1996)

11. Hong C., H.A.Kim, G.L.Firestone & L.F.Bjeldanes: 3,3'-Diindolylmethane (DIM) induces a G(1) cell cycle arrest in human breast cancer cells that is accompanied by Sp1-mediated activation of p21(WAF1/CIP1) expression. *Carcinogenesis* 23,1297-1305 (2002)

12. Hong C., G.L.Firestone & L.F.Bjeldanes: Bcl-2 family-mediated apoptotic effects of 3,3'-diindolylmethane (DIM) in human breast cancer cells. *Biochem Pharmacol* 63,1085-1097 (2002)

13. Chinni S.R., Y.Li, S.Upadhyay, P.K.Koppolu & F.H.Sarkar: Indole-3-carbinol (I3C) induced cell growth inhibition, G1 cell cycle arrest and apoptosis in prostate cancer cells. *Oncogene* 20,2927-2936 (2001)

14. Chinni S.R. & F.H.Sarkar: Akt inactivation is a key event in indole-3-carbinol-induced apoptosis in PC-3 cells. *Clin Cancer Res* 8,1228-1236 (2002)

15. Li Y., X.Li & F.H.Sarkar: Gene expression profiles of I3C- and DIM-treated PC3 human prostate cancer cells determined by cDNA microarray analysis. *J Nutr* 133,1011-1019 (2003)

16. Chang F., J.T.Lee, P.M.Navolanic, L.S.Steelman, J.G.Shelton, W.L.Blalock, R.A.Franklin & J.A.McCubrey: Involvement of PI3K/Akt pathway in cell cycle [illegible]gression[illegible] apoptosis, and neoplastic transformation: a [illegible]rget for cancer chemotherapy. *Leukemia* 17,590-603 (200[illegible])

17. [illegible]ara J.A., E.Casado, J.De Castro, P.Cejas, C.Belda-Iniesta[illegible] [illegible]lez-Baron: PI3K/Akt signalling pathway and cancer. *Cancer Treat Rev* 30,193-204 (2004)

18. Kucharczak J., M.J.Simmons, Y.Fan & C.Gelinas: To be, or not to be: NF-kappaB is the answer--role of Rel/NF-kappa[illegible] in the regulation of apoptosis. *Oncogene* 22,8961-8982 (2003)

19. [illegible]. & B.[illegible]Aggarwal: Nuclear factor-kappa B and [illegible]ncer: its role in prevention and therapy. *Biochem [illegible]* 64,983-888 (2002)

20. Beg A.A. & D.Baltimore: An essential role for NF-kappaB in preventing TNF-alpha-induced cell death. *Science* 274,782-784 (1996)

21. Beg A.A., W.C.Sha, R.T.Bronson, S.Ghosh & D.Baltimore: Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-kappa B. *Nature* 376,167-170 (1995)

22. Romashkova J.A. & S.S.Makarov: NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling. *Nature* 401,86-90 (1999)

23. Gustin J.A., O.N.Ozes, H.Akca, R.Pincheira, L.D.Mayo, Q.Li, J.R.Guzman, C.K.Korgaonkar & D.B.Donner: Cell type-specific expression of the IkappaB kinases determines the significance of phosphatidylinositol 3-kinase/Akt signaling to NF-kappa B activation. *J Biol Chem* 279,1615-1620 (2004)

24. Ozes O.N., L.D.Mayo, J.A.Gustin, S.R.Pfeffer, L.M.Pfeffer & D.B.Donner: NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase. *Nature* 401,82-85 (1999)

25. Li Y. & F.H.Sarkar: Inhibition of nuclear factor kappaB activation in PC3 cells by genistein is mediated via

Akt signaling pathway. *Clin Cancer Res* 8,2369-2377 (2002)
26. Cardone M.H., N.Roy, H.R.Stennicke, G.S.Salvesen, T.F.Franke, E.Stanbridge, S.Frisch & J.C.Reed: Regulation of cell death protease caspase-9 by phosphorylation. *Science* 282,1318-1321 (1998)
27. Brunet A., A.Bonni, M.J.Zigmond, M.Z.Lin, P.Juo, L.S.Hu, M.J.Anderson, K.C.Arden, J.Blenis & M.E.Greenberg: Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* 96,857-868 (1999)
28. Yang E., J.Zha, J.Jockel, L.H.Boise, C.B.Thompson & S.J.Korsmeyer: Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. *Cell* 80,285-291 (1995)
29. Pastorino J.G., M.Tafani, R.J.Rothman, A.Marcinkeviciute, J.B.Hoek, J.L.Farber & A.Marcineviciute: Functional consequences of the sustained or transient activation by Bax of the mitochondrial permeability transition pore. *J Biol Chem* 274,31734-31739 (1999)
30. Datta S.R., H.Dudek, X.Tao, S.Masters, H.Fu, Y.Gotoh & M.E.Greenberg: Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* 91,231-241 (1997)
31. Rusinol A.E., D.Thewke, J.Liu, N.Freeman, S.R.Panini & M.S.Sinensky: AKT/protein kinase B regulation of BCL family members during oxysterol-induced apoptosis. *J Biol Chem* 279,1392-1399 (2004)
32. Kane L.P., V.S.Shapiro, [illegible] & A.Weiss: Induction of NF-kappaB by the Akt/PKB kinase. *Curr Biol* 9,601-604 (1999)
33. Factor V., A.L.Oliver, G.R.Panta, S.S.Thorgeirsson, G.E.Sonenshein & M.Arsura: Roles of Akt/PKB and IKK complex in constitutive induction of NF-kappaB in hepatocellular carcinomas of transforming growth factor alpha/c-myc transgenic mice. *Hepatology* 4,[illegible]41 (2001)
34. Haefner B.: NF-kappa B: breaking a major culprit in cancer. *Drug Discov Today* 7,653-663 (2002)
35. Hideshima T., D.Chauhan, P.Richardson, C.Mitsiades, N.Mitsiades, T.Hayashi, N.Munshi, L.Dang, A.Castro, V.Palombella, J.Adams & K.C.Anderson: NF-kappa B as a therapeutic target in multiple myeloma. *J Biol Chem* 277,16639-16647 (2002)
36. Orlowski R.Z. & A.S.Baldwin, Jr.: NF-kappaB as a therapeutic target in cancer. *Trends Mol Med* 8,385-389 (2002)
37. Biswas D.K., S.C.Dai, A.Cruz, B.Weiser, E.Graner & A.B.Pardee: The nuclear factor kappa B (NF-kappa B): a potential therapeutic target for estrogen receptor negative breast cancers. *Proc Natl Acad Sci U S A* 98,10386-10391 (2001)
38. Rahman K.M., O.Aranha & F.H.Sarkar: Indole-3-carbinol (I3C) induces apoptosis in tumorigenic but not in nontumorigenic breast epithelial cells. *Nutr Cancer* 45,101-112 (2003)

Key Words: DIM, Akt, NF-kappaB, Apoptosis, Prostate, Cancer

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<Abstract>Prostate cancer is the second
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in men in the United States. I3C and
its in vivo dimeric product, DIM, have
been found to inhibit the growth of
prostate cancer cells. However, the
molecular mechanism(s) by which DIM
elicits its effects on prostate cancer
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